VIBRIO VULNIFICUS DYNAMICS IN A SOUTH TEXAS BAY

A Thesis

by

SHELLI LEE MEYER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2007

Major Subject: Oceanography

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Approved by:

Co-Chairs of Committee,	Richard A. Long
	Daniel C.O. Thornton
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	John R. Schwarz
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ABSTRACT

Vibrio vulnificus Dynamics in a South Texas Bay. (August 2007)

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Vibrio vulnificus is a human pathogen commonly found in coastal and estuarine waters in temperate and subtropical regions across the world. The ecology of *V. vulnificus* has been studied in these regions primarily using cultured-based methods for enumeration of *V. vulnificus* from the environment. Optimal temperature and salinity ranges have been established, but relationships with other environmental parameters have not been studied as extensively. The primary objective of this study was to better understand the ecology of *V. vulnificus* and how environmental parameters found in south Texas bays and estuaries regulate its distribution.

A recently developed molecular biological technique for the direct enumeration of *V. vulnificus* from estuarine water column samples was used to test three hypotheses: 1) *V. vulnificus* makes up a greater percentage of the total bacterial population in the water column under low oxygen conditions; 2) Powderhorn Lake serves as a point-source for *V. vulnificus* in Matagorda Bay; 3) Higher *V. vulnificus* concentrations are found in the water column when oyster reefs are present. These hypotheses were formed to improve predictive models, identify potential hot-spots for *V. vulnificus* in the water column, and to better inform stakeholders as to when and where risk of infection might be greatest.

Dissolved oxygen was rarely low enough in the environment to stress aerobic bacteria in the water column, so the first hypothesis could not be appropriately tested. Neither higher concentrations nor detection frequencies of *V. vulnificus* were found in Powderhorn Lake compared to the rest of the bay, so Powderhorn Lake was not identified as a point-source for *V. vulnificus.* Higher concentrations and detection frequencies of *V. vulnificus* were not found at sites with oyster beds, so oyster beds

cannot be used as indicators of higher concentrations of *V. vulnificus* in the water column.

Interestingly, patchiness of *V. vulnificus* was observed temporally and spatially throughout the sampling region of Matagorda Bay, on a scale that has not been frequently examined. Variation occurred between samples in close proximity to one another, as well as between sampling dates. This distribution exhibited a small scale patchiness not frequently reported in past studies.

ACKNOWLEDGMENTS

I thank my advisor Richard A. Long for his guidance, patience and support, and my co-chair Dr. Daniel C. O. Thornton for his guidance and feedback. I also thank my committee members, Drs. Kai E. Elgethun and John R. Schwarz for their input and suggestions.

I thank Dr. Laurie Davidson for use of facilities and equipment and guidance for the utilization of instrumentation.

I thank Mike Powell for assistance in selecting sampling sites and sample collection. I thank Erin Anitsakis for her help with laboratory techniques and for helping to collect samples many times. I also thank Chrissy Weiderwohl and Laura Rubiano-Gomez for helping to sample multiple times. A long list of others also helped to collect samples: Valeria Kiselkova, Rob Hetland, Gabe Burns, Alfonso Franco, Lindsey Visser, Julia O'Hern, Caroline Wilson, Katie Fosha, and Mike Navatta. A special thanks also to Adam Marchand for helping to generate the maps used to depict the sampling sites for this research. I thank all for their help, friendship and support.

Throughout this project I received support from the National Science Foundation, Environmental Protection Agency-Formosa Plastics Environmental Trust Fund and the National Institute of Health-Texas A&M University's Center for Environmental and Rural Health grants awarded to R.A.L., the Texas Water Resources Institute Mills Scholarship program, and the Department of Oceanography at Texas A&M University.

I also thank my family and friends for their support through this work. I thank each of them for their encouragement, love and support.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I
INTRODUCTION

PROJECT OBJECTIVE

Simply swimming or fishing in the bays and estuaries along the Gulf of Mexico puts even healthy people at risk of a potentially deadly bacterial infection if they have even a small open wound or cut. Annually, there are 35 deaths in the United States due to *Vibrio vulnificus* infections (Ho et al., 2003). Even more infections lead to surgical debridement, amputations of limbs (Bisharat et al., 1999), and or prolonged antibiotic treatment. These infections impact local economies as recreational water users are frightened away.

The primary objective of this study is to understand the ecology of *V. vulnificus* and how environmental parameters found in south Texas bays and estuaries regulate its distribution. Temperature and salinity have been well studied and observed as the primary variables correlating with *V. vulnificus* distribution and abundance (Kelly, 1982; Tamplin et al., 1982; Oliver et al., 1983). Geographic location has been observed to contribute to which variable is dominant in the ecology of *V. vulnificus* (Wright et al., 1996; Lipp et al., 2001; Fukushima and Seki, 2004). Although temperature and salinity are well studied, other parameters remain to be considered as extensively, such as dissolved organic matter, pH effects (Lipp et al., 2001), or dissolved oxygen concentration. This project seeks to evaluate the effects of the aforementioned environmental parameters on the abundance and distribution of *V. vulnificus* in Texas estuarine waters.

BACKGROUND

V. vulnificus Characteristics and Pathogenicity

V. vulnificus is classified as a facultative anaerobic gram-negative rod. It thrives in the marine environment, and has been isolated from oysters, eels, sediments, and the

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This thesis follows the style of *Environmental Microbiology*.

water column. *V. vulnificus* is most often recognized because of its pathogenicity towards humans and farm-raised eels. A distinction has been made between various strains of *V. vulnificus* by grouping similar strains together into different biotypes initially based on differing phenotypes, serological properties, and host range (Tison et al., 1982).

Three different biotypes have been identified to date. Biotype 1 is an opportunistic human pathogen that is commonly isolated from the environment and clinical samples (Biosca et al., 1997; Hoi et al., 1998; Watanabe et al., 2004). The eel pathogen responsible for most eel mortality in aquaculture belongs to biotype 2 (Tison et al., 1982; Esteve-Gassant and Amaro, 2004). This biotype is primarily cultured from eels or humans after handling eels, and has rarely been isolated from the environment (Biosca et al., 1997; Arias et al., 1998; Hoi et al., 1998; Esteve-Gassant and Amaro, 2004; Marco-Noales et al., 2004). *V. vulnificus* isolated from human wound aspirates was distinguishable from both aforementioned biotypes (Bisharat et al., 1999), lending to the recognition of biotype 3.

Two primary routes of *V. vulnificus* infections are through ingestion and the exposure of open flesh wounds. The consequent effects are septicemia and wound infections sometimes leading to necrotizing fasciitis (Janda et al., 1988; Bisharat et al., 1999). Septicemia, or blood poisoning, occurs when toxin-producing bacteria proliferate in the bloodstream. The highest percentage of individuals who experience septicemia suffer from chronic liver disease (Haq and Dayal, 2005), followed by people with compromised immune systems and other chronic diseases such as diabetes (Strom and Paranjpye, 2000; Ho et al., 2003; Kuhnt-Lenz et al., 2004; Haq and Dayal, 2005). Necrotizing fasciitis is an infection that causes inflammation and destruction of deeper layers of the skin.

There are almost 76 million illnesses attributed to foodborne disease in the United States each year (Mead et al., 1999). Annually in the U.S., shellfish consumption leads to as many as 1.3 million bacterial illnesses, of which twenty percent are caused by *Vibrio* species (Butt et al., 2004). Non-cholerae vibrios are responsible for the highest

percentage of death (95%) caused by shellfish borne disease (Wittman and Flick, 1995). *V. vulnificus* is the second most commonly associated vibrio with human disease in the U.S. (Butt et al., 2004) and has the highest fatality rate among all foodborne pathogens (Mead et al., 1999).

From 1988 to 1996, out of the 422 reported *V. vulnificus* infections in the United States, 45% were due to wound infection, 43% were primary septicemia after ingestion, with the remaining from gastroenteritis and undetermined contact (Strom and Paranjpye, 2000). Fatality rates for ingestion are much higher than rates for wound infection, over 50% and 25% respectively (Chen et al., 2004; Fukushima and Seki, 2004). In Florida, consumers suffering from liver disease and eating raw oysters are 92 times more likely to become infected than consumers without liver disease, and are 190 times more likely to die of infection (Wittman and Flick, 1995).

While the infective dose for ingestion in humans is not established, Jackson et al. (1997) estimated the infective dose at 1-6 x 10^5 *V. vulnificus* cells in susceptible individuals. This was determined using case studies and quantifying *V. vulnificus* isolated from oysters from the implicated restaurant. Blood samples from the fatalities showed levels from $8.8x10^8$ to $2.0x10^{10}$ colony forming units (CFU's) per milliliter of blood by plating 10-fold serial dilutions (Jackson et al., 1997). A lethal dose for some strains of *V. vulnificus* has been found as low as 10 CFU's in mouse models (Starks et al., 2000). Infection is rapid, sometimes causing death within 1-2 days after infection (Brasher et al., 1998).

Contact of an open wound with contaminated seawater or marine organisms may also cause infection regardless of preexisting chronic illnesses (Tacket et al., 1984; Wittman and Flick, 1995; Bisharat et al., 1999; Ulusarac and Carter, 2004), although most infections in healthy individuals are acute. Exposure may lead to severe necrotizing wound infections, as well as septicemia in more susceptible patients (Kuhnt-Lenz et al., 2004). While the fatality rate is considerably lower with wound infections, many infections result in surgical removal of the infection. In Israel from 1996-97, there were 62 cases of wound infection and septicemia due to *V. vulnificus*. Approximately

66% of the patients underwent surgical debridement to eliminate infection, nearly 12% had finger amputations and one patient endured a total limb amputation (Bisharat et al., 1999).

There are several factors that have shown potential for contributing to the virulence of *V. vulnificus.* Surface factors include the presence of a capsular polysaccharide (CPS) that surrounds the cell. This provides the cells with protection from bactericidal mammalian serum as well as phagocytosis by macrophages (Yoshida et al., 1985; Simpson et al., 1987; Strom and Paranjpye, 2000). Lipopolysaccharides may also contribute to virulence through the induction of inflammation, tissue damage, and septicemic shock (Strom and Paranjpye, 2000). The presence of pili allow for biofilm formation and attachment of *V. vulnificus* cells to epithelial surfaces, possibly contributing to virulence as well (Paranjpye and Strom, 2005).

Extracellular toxins are considered to be strong virulence factors. Extracellular cytolysin is a hemolysin that acts as a vascular permeability factor that ultimately causes host cell death (Rho et al., 2002) and virulence has been correlated with the amount of cytolysin produced during cell growth (Kreger and Lockwood, 1981). A second hemolysin has been identified as a virulence factor in mice (Chen et al., 2004). *V. vulnificus* metalloprotease increases vascular permeability by stimulating the generation of inflammatory mediators, resulting in septicemia accompanied by edematous lesions (Miyoshi and Shinoda, 2000; Miyoshi et al., 2004). Other toxins and enzymes that have been mentioned as possible virulence factors are enterotoxin, chitinase, and phospholipase (Strom and Paranjpye, 2000).

Contributions to virulence are not limited to the pathogen alone. The host may also make a significant contribution to the virulence of *V. vulnificus*. Iron has been found to be a limiting factor in the ability of *V. vulnificus* to survive and grow within mammalian sera. Without an elevated concentration of iron, such as what may be provided in patients suffering from hemachromatosis, a condition that increases the saturation of transferrin with iron, *V. vulnificus* cells would die. An intravenous

injection of iron into mice decreased the LD_{50} of *V. vulnificus* cells from 10^6 to 1.1 cells (Wright et al., 1981).

V. vulnificus Ecology

Vibrios are found in aquatic environments such as estuaries, marine coastal waters and sediments, and aquatic culture settings in temperate and tropical climates around the world (Thompson et al., 2004). *V. vulnificus* has been isolated from estuaries, coastal waters, sediments and oysters in Japan (Fukushima and Seki, 2004), China (Chan et al., 1986), India (Parvathi et al., 2004), Israel (Bisharat et al., 1999) France (Hervio-Heath et al., 2002), Italy (Barbieri et al., 1999), Denmark (Hoi et al., 1998), both the West and East coasts of United States (Kaysner et al., 1987; Wright et al., 1996; Pfeffer et al., 2003), and numerous studies along the coast of the Gulf of Mexico (Kelly, 1982; Tamplin et al., 1982; Motes et al., 1998; Lin et al., 2003; Panicker et al., 2004).

Literature focusing on the ecology of *V. vulnificus* typically reports that either salinity, temperature, or both are the most important variables when considering variations of abundance and distribution of *V. vulnificus* in oysters and seawater. Ideal conditions for *V. vulnificus* have been observed at temperatures ranging from 18-30°C and salinities between 6-16 (Kelly, 1982; Tamplin et al., 1982; Oliver et al., 1983; Kaysner et al., 1987; Kaspar and Tamplin, 1993; Pfeffer et al., 2003). Isolation of the organism has occurred outside of these ranges in much lower numbers and frequency.

Several studies reported difficulty recovering isolates when temperatures fell below 15°C (Kelly, 1982; Kaysner et al., 1987; Lin et al., 2003; Lin and Schwarz, 2003; Pfeffer et al., 2003). This is often attributed to the ability of an organism to enter the viable but non-culturable state (VBNC) when environmental conditions are not ideal. Under these circumstances the cells are still living, but it is not possible to culture cells on traditional media in a laboratory. Cells can enter into this state at temperatures between 10 and 15°C within 5-7 days, and they can be resuscitated after 1-4 days at 22-24°C (Oliver et al., 1995). There is still debate, however, as to whether this is actually

from cells being resuscitated or if the few remaining culturable cells initiate growth of cells back to detectable levels.

Despite the observed difficulty in isolation at low temperatures, one study in Japan was successful in isolating *V. vulnificus* year-round regardless of temperature ranging from 5-32°C, as well as salinities below 5 (Fukushima and Seki, 2004). Geographical location plays a significant role in determining the dominant variable affecting distribution patterns of *V. vulnificus*. One review illustrated that *V. vulnificus* is found in oysters along the US East, West, and Gulf coastlines, the highest concentrations usually found on the Gulf Coast and Southeast coastline (Wittman and Flick, 1995). Between 1984 and 1993, 23% of shellfish borne disease and 41% of deaths were caused by consumption of oysters from the Gulf of Mexico (Wittman and Flick, 1995). The following case studies demonstrate the differences in variable importance relative to geographic location in the United States.

Temperature is responsible for a majority of *V. vulnificus* variability in oysters from the Gulf of Mexico (Motes et al., 1998). In Galveston Bay, *V. vulnificus* isolation has been positively correlated with water temperature in both oysters and the water column. There was a slight but significant negative correlation between *V. vulnificus* concentration and salinity in the water column, but no correlation in oysters (Lin et al., 2003). Dissolved oxygen was not considered in this study.

Others observed no significant correlation between temperature and concentrations of *V. vulnificus* in bay waters. A significant inverse correlation was found between salinity and *V. vulnificus* concentration in Chesapeake Bay water samples. However, there was not a significant correlation between water temperature and *V. vulnificus* concentrations in warmer months when temperatures ranged from 8-26°C (Wright et al., 1996). While dissolved oxygen was measured, there was no correlation analysis conducted.

Lipp et al. (2001) determined that salinity was the most important variable determining the geographical distribution of *V. vulnificus* in the water column of subtropical regions of the Gulf of Mexico. While a positive correlation was observed

with temperature and isolated *V. vulnificus* concentrations, salinity was determined to be a stronger factor than temperature in determining prevalence of *V. vulnificus* in Charlotte Harbor, Fl. There were no observations of dissolved oxygen concentration. Lipp et al. (2001) concluded that their work supports that the effects of temperature and salinity are interrelated and region specific.

Pfeffer et al. (2003) conducted their studies in the water column and sediments of a North Carolina estuary. They observed a slight negative correlation with dissolved oxygen concentration in the water column with the isolation of *Vibrio* species and *V. vulnificus;* however, dissolved oxygen did not contribute to the variability of *V. vulnificus* in the water column. The correlation was in part attributed to the relationship of rising temperatures to decreasing oxygen saturation in the water column. This study established a significant relationship between *V. vulnificus* and temperature, accounting for almost 50% of the variability, similar to the findings of Motes et al. (1998) in Gulf Coast oysters. The range of salinity in which *V. vulnificus* were isolated from was 8-14, but no significant correlation was determined (Pfeffer et al., 2003).

Two strains of *V. vulnificus* isolated from both oysters and bay water were identified in Galveston Bay, TX. No *V. vulnificus* were recovered during the coldest months, December through February when temperatures fell below 15°C (Lin et al., 2003; Lin and Schwarz, 2003). The strains varied in their temporal isolation, only one of which was isolated in March through May, and the other becoming dominant in September through the remainder of the year. The difference in the predominant strain could be attributed to the higher salinities experienced later in the season. Throughout the study, *V. vulnificus* was isolated from 74% of oyster samples, and only 45% of water samples (Lin et al., 2003). No analysis of dissolved oxygen concentration was carried out in this study.

The primary environmental variable affecting *V. vulnificus* distribution and abundance is in large part due to the geographic location. Temperature displays different degrees of importance when considering different systems, e.g., Chesapeake Bay versus a Gulf Coast estuary. Salinity also plays a varying role depending on

location of the study. Although the negative correlation between dissolved oxygen concentration and *V. vulnificus* isolation was not deemed statistically significant in a North Carolina estuary (Pfeffer et al., 2003), it does not imply that it cannot be significant in a different region. There is growing evidence that environmental variables impart a different level of effect on *V. vulnificus* ecology depending on geographical location. This study will examine the interrelated effects of temperature, salinity, and dissolved oxygen concentration on the ecology of *V. vulnificus.*

STUDY LOCATION

In the summer of 2004, the Victoria Advocate newspaper (Victoria, TX) reported several Vibrio infections, including infections caused by *V. vulnificus*, in South Texas. A few of the stories covered a fatality in which a man was infected after fishing near Port O'Connor, TX. Another man became infected through an open cut after fishing in Matagorda Bay and Baffin Bay, TX. That same year there were three reported deaths and several amputations among the 20 reported cases in Texas. In 2005, there were eleven reported cases of Vibrio infection in South Texas by the end of July, four from wound exposure, three from raw oyster consumption, and four from unknown causes (Victoria Advocate). In the summer of 2006, no infections were reported in the local Texas newspaper.

Estuaries are important natural resources to both the people who rely on them for work or pleasure, and also the delicate ecosystem that exists within. Healthy people merely swimming in the bay could put themselves at risk of a serious infection. Fishermen often wade fish and put themselves at greater risk by not wearing waders during the warmest times of the season, when several studies have shown *V. vulnificus* numbers peak. It is important to understand the dynamics of an estuarine system to protect the life inhabiting the bay as well as the users. Improving our understanding of possible natural predictors can help maintain the safety of those who rely on the bay system.

For this study Matagorda Bay, an estuary located on the southern Gulf Coast of Texas, was chosen as a good study site offering the potential to exhibit a gradient of environmental variables. Port O'Connor, a small town near the Gulf of Mexico inlet to Matagorda Bay, is implicated in several contracted infections of *V. vulnificus*. Powderhorn Lake is adjacently connected to the bay and serves as a junction between the bay and the freshwater sources of Coloma Creek and Coloma Lake. Powderhorn Lake was the most likely region to exhibit stratification and was also said to be stagnant during the summer, possibly leading to low oxygen conditions.

The overarching goal of this study is to better understand environmental variables that regulate *V. vulnificus* ecology to improve predictive models. Specific objectives within this framework are 1) examine *V. vulnificus* ecology over environmental gradients, i.e. temperature, salinity, and oxygen; 2) Determine potential point source(s) of *V. vulnificus* within the system and 3) Improve predictive models for policy makers and managers.

HYPOTHESES

Vibrio vulnificus is a facultative anaerobe, making it more versatile than other naturally occurring marine bacterial species. Based on this knowledge the first hypothesis is:

$H1_o$: Under low oxygen conditions, *V. vulnificus* does not make up a greater percentage of total bacteria in the water column.

$H1_a$: Under low oxygen conditions, *V. vulnificus* makes up a greater percentage of total bacteria in the water column.

Powderhorn Lake serves as a junction between Coloma Creek and Coloma Lake to Matagorda Bay. This freshwater inlet makes Powderhorn Lake more likely to exhibit greater stratification than the rest of Matagorda Bay. Higher concentrations of organic material are also expected to exist within this lake system.

$H2_o$: Powderhorn Lake does not serve as a point source for *V. vulnificus.*

$H2_a$: Powderhorn Lake serves as a point source for *V. vulnificus.*

Oyster beds are common throughout Matagorda Bay. As filter-feeders, oysters concentrate small particles from the water-column including bacteria, and of primary interest, *V. vulnificus*. One study observed that sediments surrounding oyster beds were more likely to contain *V. vulnificus* than sediments that lack oysters (Wright et al., 1996). The exchange of water between the oysters and the surrounding water column presents the possibility that cells concentrated within the oyster are released back into the water column.

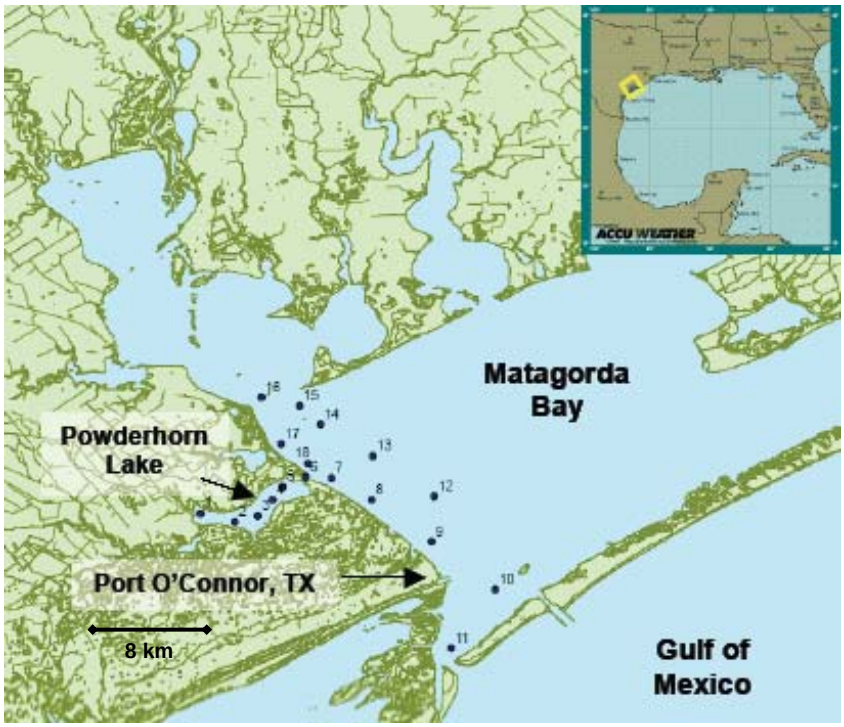
$H3_o$: Higher concentrations of *V. vulnificus* do not exist at sites where oyster beds are present.

$H3_a$: Higher concentrations of *V. vulnificus* exist at sites where oyster beds are present.

CHAPTER II
MATERIALS AND METHODS

SAMPLE COLLECTION

A spatial and temporal study of *V. vulnificus* was conducted in Matagorda Bay, Texas. Sites were distributed throughout the west end of the bay system after consultation with a local fisherman (See Fig. 2.1). Powderhorn Lake (sites 1-5), near-shore (sites 7-9, 16-18) and bay samples (sites 10-16) were collected from a shallow water fishing boat (Fig. 2.1). Water column samples were collected over an 18 month period from April 2005 to October 2006. Samples were collected twice per month from June through October of 2005 as concentrations of *V. vulnificus* are generally higher



Site #	Coordinates N28*	W096*
1	28.799	33.309
2	28.978	32.256
3	29.579	30.823
4	30.046	29.916
5	30.449	29.313
6	30.771	28.966
7	30.560	28.683
8	30.231	28.001
9	28.757	25.982
10	27.014	22.852
11	23.280	23.587
12	29.504	25.397
13	30.818	27.377
14	31.521	28.377
15	33.208	30.532
16	33.589	31.798
17	31.678	30.047
18	31.110	28.890

Fig. 2.1. Sampling sites in Matagorda Bay, Texas with corresponding GPS coordinates. Nineteen sites were sampled each trip, 18 throughout the bay and one at a marina in Port O'Connor, TX, April 2005 – October 2006.

during the summer than the winter (Lipp et al., 2001; Lin et al., 2003), and only once per month from October 2005 through October 2006 (once per month in the summer of 2006 due to limited funding).

Physico-chemical parameters were measured first at each site using a Manta Water Quality Multiprobe™ and the Amphibian™ data logger (Eureka Environmental Engineering, Austin, TX). Measurements taken include depth (pressure), temperature, dissolved oxygen concentration (percent dissolved oxygen saturation), salinity (conductivity), pH, and Oxidation Reduction Potential. Water samples were then collected using Just Another Water Sampler (JAWS). JAWS is a vertical water sampler constructed out of PVC pipe, nylon rope, a nozzle and a rubber ball. The device was constructed to allow water to fill from the bottom, and hold the water as JAWS is pulled back to the surface. One sample was collected mid-column from shallow water columns (<1 m) and surface and bottom samples were collected from deep water columns (>1 m) and where strong salinity gradients were observed. Samples were stored in acid washed 1 L polypropylene collection bottles, stored on ice and transported back to Texas A&M University.

BACTERIAL ENUMERATION

Five ml of each sample were fixed with 0.22 μm filtered borate buffered formalin at a 2% final concentration at 4°C for at least one hr but no longer than 24 hrs. A two ml sub-sample was stained with 4', 6'-diamidino-2-phenylindole (DAPI, Sigma; 1 μg ml^{-1} final concentration) and incubated in the dark for at least 15 minutes. Stained samples were concentrated onto black polycarbonate membrane filters (Millipore, 25 mm diameter, 0.22 μm; Porter and Feig, 1980) which were mounted onto glass microscope slides and stored at -20°C (Turley and Hughes, 1992).

Bacterial counts were carried out on a Zeiss Axioplan Imaging 2 microscope (Zeiss filter set 02; G 365/ LP 420). Bacterial counts were determined using a minimum of 200 cells and seven fields or up to 20 fields per slide for statistical validity (Kirchman et al., 1982). Total bacterial abundance was calculated using the following equation:

$$a = x * 1/v * cf$$

a = abundance

x = average number of bacterial cells per field

v = volume of sample filtered

cf = conversion factor for (filter surface area)/(ocular grid surface area)

DNA EXTRACTION

Samples for DNA extraction were prepared immediately upon return to the laboratory. Between 50-200 ml of sample water was filtered onto 25 mm dia., 0.2 μm pore size Supor® 200 polyethersulfone membrane filters (Pall Corp.). Volumes were recorded, and filters were stored at -80°C. DNA was extracted using a modification of Boström et al. (2004), a protocol designed for Q-PCR analysis of small sample volumes.

Filters were cut into thin strips before beginning extraction using a sterile razorblade and forceps. Following Boström et al. (2004), cells trapped on the filter were lysed in 525 μl of cell lysis buffer (400 mM NaCl, 750 mM Sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris-HCl pH 9.0) and 10 μl of freshly prepared lysozyme (1.1 mg ml^{-1}, Sigma-Aldrich). Samples were incubated at 37°C for 30 minutes. Next, 3 μl of proteinase K (100 μg ml^{-1}) and 60 μL of sodium dodecyl sulfate (1%) were added. Samples were incubated at 55°C for 12 hrs rotating at approximately 5 rpm.

Samples were passed through silica membrane UniPrep Spin Filters (Genesee Scientific) to reduce the amount of detritus and suspended sediments carried over. Filters were rinsed with 500 μl of TE (10 mM Tris-HCl pH 8.0, 1mM EDTA), passed through the spin column and pooled with the lysate. DNA was precipitated using 1/10 volume 3 M sodium acetate, 0.6 volume cold isopropanol, and 1 μl glycogen (20 mg ml^{-1}) as a coprecipitant. Glycogen was used in place of the Baker's yeast tRNA used by Boström et al. (2004) as tRNA interfered in PicoGreen DNA quantitation while the glycogen did not (see below). Samples were precipitated at -20°C for > 60 min. Samples were then centrifuged at 20,000 g at 4°C for 20 min, the pellet washed with iced

70% molecular grade ethanol and centrifuged again. The ethanol was decanted and the samples dried. Samples were resuspended in 50 μl of PCR grade water and stored at -20°C.

DNA quantitation was carried out on preliminary DNA extractions from known amounts of cultured *V. vulnificus.* DNA yields were over four times greater than expected, so the effect of tRNA on the PicoGreen assay was tested. All five concentrations of tRNA tested (10 – 50 μg) gave a strong fluorescent signal. This accounted for the higher than expected yields of DNA.

Glycogen has been used as an alternative coprecipitant in environmental DNA extractions (Tsai and Rochelle, 2001), so glycogen was also examined for interference with fluorescence using the PicoGreen protocol. Glycogen was tested by adding different concentrations to a standard curve of DNA. The addition of glycogen to the standard curve did not show a statistically significant difference in fluorescent signal using a two-tailed t-test ($p>0.05$). 50 μg of tRNA was subsequently replaced with 20 μg of glycogen as a coprecipitant.

Total DNA was quantified using Quanti-iT™ PicoGreen® dsDNA Reagent (Molecular Probes) following the manufacturer's instructions for 200 μl volumes. A standard curve was prepared according to manufacturer's protocol for a high-range standard curve. Concentrations were determined on a SpectraMax Gemini EM spectrofluorometer (Molecular Devices; Excitation/Emission - 480/520). Samples were diluted to 10 ng μl^{-1} for further analysis.

DNA quality was tested by PCR amplification of a 1465 base-pair region of the 16S rDNA using 10 ng of environmental DNA. The primers used were 27F and 1492R (Sigma Genosys), 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GGYTAC CTTGTTACGACTT-3' respectively (Weisburg et al., 1991). Bovine Serum Albumin (BSA) has been shown to relieve inhibition of PCR due to humic acids (Kreader, 1996), and was required for positive amplification of the environmental DNA. Taq polymerase Mastermix (Qiagen) chemistry was used for a 20 μL PCR reaction, adding equimolar primer concentrations (500 nM final concentration), BSA (300 μg mL^{-1} final

concentration) and 10 ng of DNA template. Touchdown PCR was performed with initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 3 min, 65°C for 1 min (-0.5°C each cycle), and 72°C for 2 min followed by 5 additional cycles annealing at 50°C, and a final extension at 72°C for 10 min (MJ Research PTC-200). Approximately 90% of all of the environmental samples were positively amplified.

QUANTITATIVE POLYMERASE CHAIN REACTION (Q-PCR)

Q-PCR, also referred to as real-time PCR, was applied to environmental DNA samples to detect and quantify *V. vulnificus* by targeting the *V. vulnificus* hemolysin (*vvh*) gene. A region of the *vvh* gene is amplified by PCR, exponentially increasing the PCR product with each cycle. SYBR Green I is a fluorescent double-stranded DNA specific stain (Rasmussen et al., 1998). An increase in the target gene is detected by an increase in fluorescent signal. The concentration of the targeted gene is determined when the fluorescence reaches an empirically determined threshold. The PCR cycle at which the fluorescence reaches the threshold is the threshold cycle (C_T). The sample concentration is calculated by comparing the unknown C_T value to a set of C_T values based upon a standard curve.

Target Gene and Primers

The *vvh* gene was selected as a target because it appears to be a conserved and ubiquitous cytolysin gene among *V. vulnificus* strains regardless of isolation source (Wright et al., 1985). The *vvh* primers from Panicker et al. (2004) and Gonzalez et al. (2004) were compared *in silico* using the NCBI BLASTn search tool against the GenBank database (November 2006; http://www.ncbi.nlm.nih.gov/BLAST/). The primer set from Panicker et al. (2004) showed a greater number of corresponding *V. vulnificus* entries. In addition, Panicker et al. (2004) showed the primer set was sensitive and 100% specific for *V. vulnificus* using isolates from various sources. The primers used are:

L-vvh – 5’-TTCCAACTTCAAACCGAACTATGA-3’

R-vvh – 5'-ATTCCAGTCGATGCGAATACGTTG-3'

Primers were purchased through Integrated DNA Technologies, Inc. (Coralville, IA). The primers amplify a 205 base pair segment between nucleotides 786-990 of the *vvh* gene (GenBank accession no. M34670; Panicker et al. 2004).

Optimization

Q-PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System, available through the Genomics Facility in the Center for Environmental Research and Health at Texas A&M. The standard pre-set cycling conditions for the ABI system were used (Table 2.1). Power SYBR® Green PCR Mastermix (Applied Biosystems), chemistry optimized for the ABI system, was used for Q-PCR. Primer concentration was optimized according to the manufacturer's instructions using DNA from *V. vulnificus* isolates extracted with the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc.). This system revealed a statistically lower C_T value (one-tailed t-test; $p<0.05$) using 300 nM concentrations of both the forward and reverse primers.

Table 2.1. Pre-set cycling parameters on the ABI 7900HT Fast Real Time PCR System. Power SYBR Green chemistry was used for Q-PCR optimization and quantitation of *V. vulnificus* in environmental samples.

STAGE	1	2	Repeat 40x's	3	Dissociation Curve	3
Temp °C	95	95	60	95	60	95
Time min:sec	10:00	:15	1:00	:15	:15	:15

PCR inhibition issues are common with environmental DNA because of carry over of humic acids and other inhibitors during the DNA extraction (Wilson, 1997; Hugenholtz and Goebel, 2001). BSA was tested in the Q-PCR chemistry to determine if it affected the efficiency of the reaction. *V. vulnificus* DNA samples were tested with and without BSA addition with the optimized primer concentration. No statistically significant differences in the C_T values were observed (two-tailed t-test; $p>0.05$).

Environmental DNA spiked with *V. vulnificus* DNA was tested in the same manner. No amplification occurred without BSA, even at the highest concentration of *V. vulnificus.* The chemistry for all subsequent Q-PCR reactions was prepared as in Table 2.2.

Table 2.2. Optimized Q-PCR chemistry. Per manufacturer's instructions, components of the chemistry were optimized. Primer concentrations were tested at 50 nM and 300 nM concentrations. Template amount was tested at concentrations from 10 – 100 ng per reaction.

	SybrGreen Q-PCR Mastermix (μL)	Forward Primer 5μM (μL) L-vvh	Reverse Primer 5μM (μL) R-vvh	BSA (μL) 10 μg μL^{-1}	Deionized Water (μL)	Template (μL) 10 ng μL^{-1}	Total Volume (μL)
Per Reaction	7.50	0.9	0.9	0.45	4.25	1.00	15.00

The concentration of environmental DNA template used in each reaction was also optimized using the chemistry as described in Table 2.2. Increasing the amount of DNA consequently increases the amount of inhibitors. Six environmental samples positive for the *V. vulnificus* target gene were tested at different concentrations. All samples gave positive signals at the lowest concentration, 10 ng of DNA per reaction; however, increasing the DNA concentration did not increase the calculated amount of *V. vulnificus* copies present as expected. Undetectable C_T values were observed in all samples with more than 50 ng of template. It is recognized that sensitivity may not be great enough to detect low numbers of *V. vulnificus* in the DNA template. As demonstrated, however, increasing the amount of template in the reaction did not increase sensitivity in this assay. Ten ng of template was used for each reaction.

Standard Curve Preparation and Testing

The standard curve was prepared using DNA extracted from *V. vulnificus* isolates obtained from the laboratory of Dr. John Schwarz at Texas A&M University, Galveston. The standard curves were prepared following the protocol provided by Applied Biosystems (http://docs.appliedbiosystems.com/pebiodocs/04371090.pdf). Two *V. vulnificus* genomes have been sequenced; *V. vulnificus* CMCP6 (5.1 million base pairs; GenBank accession no. AE0169795.2) and *V. vulnificus* YJ016 (5.26 million base pairs;

GenBank accession no. BA000037.2). A low-range average of the two was used, 5.15 Mbp, as the target genome length to calculate the number of genome copies per reaction in the standards. The mass of a single *V. vulnificus* genome was determined using the following equation:

$$M_G = L_G \times M_{bp}$$

M_G = Mass of target genome

L_G = length of target genome

M_{bp} = mass of 1 base pair = 1.096e-21

To prepare the standard curve, environmental DNA from Matagorda Bay (18Oct06) was spiked with serially diluted *V. vulnificus* DNA. Serially diluted *V. vulnificus* DNA (300,000 to 3 *V. vulnificus* genome copies) was adjusted to 10 ng μl^{-1} with environmental DNA. There was not a significant difference (two-tailed t-test; $p>0.05$; see Table 2.3) in the C_T value between *V. vulnificus* standards with background DNA and those without. A single set of standards were prepared and individual aliquots were stored at -80°C.

Table 2.3. Q-PCR results from *V. vulnificus* standards with and without background DNA. There was not a significant difference in the C_T when background DNA was added to the known *V. vulnificus* concentrations. Samples were run in duplicate.

V. vulnificus	Standards without Background DNA		Standards with Background DNA		P(T<=t) two-tail
Genome copies	C_T	C_T	C_T	C_T	
300,000	16.02	15.97	16.14	16.19	0.182
30,000	n. d.	n. d.	19.45	19.34	
3,000	23.04	23.57	23.25	23.11	0.773
300	n. d.	n. d.	26.9	26.95	
30	n. d.	n. d.	30.57	29.31	
3	32.86	und	und	und	

n. d. – not determined
und – undetectable

Dissociation curve analysis was used to determine the specificity of the products detected. The specific melting temperature in the dissociation curve was determined at

82°C, and non-specific binding occurred at 73°C. Specific peaks occurred at 82°C for the standard curves containing only *V. vulnificus* as well as those with *V. vulnificus* and environmental DNA. For quantitative purposes, only samples with 300 copies per 10 ng of template DNA were considered accurate. The melting curve analysis for the standard curve consistently showed only specific binding for $\geq$ 300 copies; however, there was inconsistent amplification or non-specific binding in standards with $\leq$ 30 copies.

The standard curve contained low *V. vulnificus* samples equivalent to approximately 10 cells mL^{-1} and 1 cell mL^{-1} of seawater. Standards at 10 cells mL^{-1} (30 copies) showed positive, specific binding 47% of the time, and showed positive, specific and non-specific binding an additional 12% of the time. *V. vulnificus* was determined to be present when specific binding occurred. Inconsistent binding between duplicates was attributed to the small amount of *V. vulnificus* DNA compared to the remaining environmental sample as shown by inconsistent binding between duplicates in the low standards with *V. vulnificus* template added. This suggests that less than 300 copies of *V. vulnificus* in a single reaction cannot be accurately quantified with this method. Standard curves were performed in duplicate for each 96 well plate.

Determination of *V. vulnificus* Concentration

V. vulnificus abundance in 10 ng of template DNA was automatically calculated with the System Software application for absolute quantitation for the ABI Real-time PCR system. *V. vulnificus* abundance in the water column was calculated by finding the ratio of *V. vulnificus* copies to total bacterial copies in the template DNA, and multiplying that ratio times the bacterial abundance for the respective sample:

$$A_{Vv} = C_{Vv}/C_{TB}*BA$$

A_{Vv} = Abundance of *V. vulnificus* → cells mL^{-1}

C_{Vv} = *V. vulnificus* copies in 10 ng of Environmental DNA

C_{TB} = Copies of total bacteria in 10 ng DNA

BA = Bacterial Abundance, determined from direct slide counts

Using this equation, it is assumed that all 10 ng of DNA used as template is bacterial DNA. *V. vulnificus* abundance estimations are likely to be low, as DNA from sources other than bacteria was extracted as well.

Concentrations of *V. vulnificus* in the water column were binned for visual representation of concentrations and distribution of *V. vulnificus* on individual maps for each sampling trip as follows:

No number = no *V. vulnificus* detected

1 = < 10 *V. vulnificus* cells mL^{-1} of water

2 = 11-100 *V. vulnificus* cells mL^{-1} of water

3 = 101-500 *V. vulnificus* cells mL^{-1} of water

4 = 501-1000 *V. vulnificus* cells mL^{-1} of water

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS (SPSS Inc, Release 14.0). A non-parametric Spearman's Correlation was used for correlations between environmental parameters and *V. vulnificus* abundance and total bacterial abundance. Multivariate analysis of variance (MANOVA) was used to determine the significance of co-variation between environmental variables and *V. vulnificus* abundance. Step-wise multiple regression analysis was used to determine prediction models for *V. vulnificus* abundance and total bacterial abundance. A Kruskal-Wallis test was used to determine significant differences in *V. vulnificus* abundance and detection between zones, depths, and locations near oyster beds. Excel t-Tests were used to determine significant differences between zones in environmental variables.

Environmental variable values were averages of 0.5 m, the length of JAWS, of the water column. A horizontal collection bottle would be better to use for shallow water columns, especially when interested in the dynamics of stratification. The total bay samples were divided into three zones for analysis; Powderhorn Lake, Near-shore, and Bay.

CHAPTER III
RESULTS

TEMPORAL AND SPATIAL VARIATION IN ENVIRONMENTAL PARAMETERS

Over the 18 month sampling period, the average water column temperature throughout the portion of Matagorda Bay sampled ranged from 17.2°C (Feb. 2006) to 31.3°C (Aug. 2005). The southwest region of Matagorda Bay was divided into three zones for analyses (Fig. 3.1); Powderhorn Lake, Near-shore, and Bay. There was not a significant spatial difference in temperature between zones ($p>0.05$), but there was a significant difference in temperature between sampling dates ($p<0.001$; Fig. 3.2A).

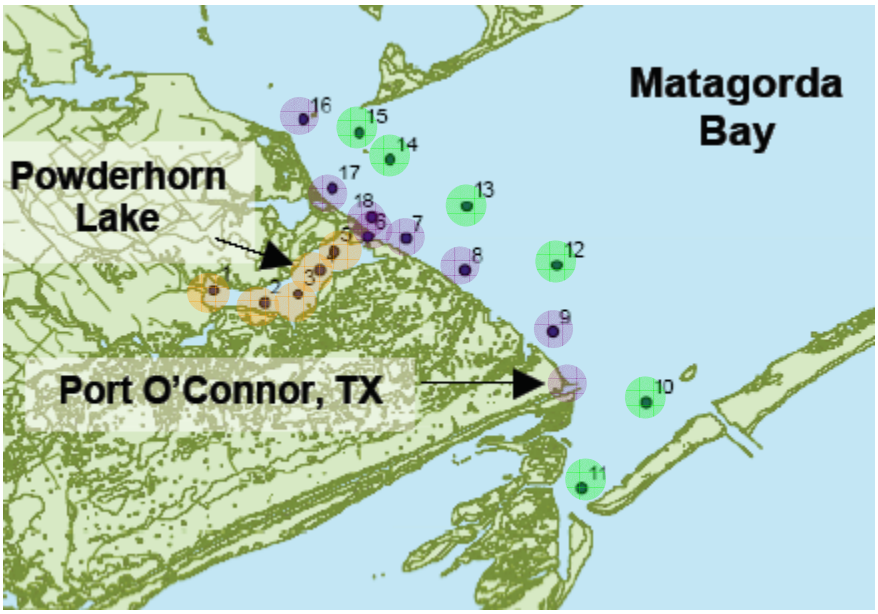


Fig. 3.1. Matagorda Bay sampling sites and zones. Sites in the Powderhorn Lake zone are highlighted in orange, in the Near-shore zone are purple, and in the Bay zone are green. Zones were selected based on the expected gradient of salinity.

A change in salinity was observed between the different zones and over time ($p<0.01$, $p<0.01$), with the lowest average salinities consistently within Powderhorn

Lake (Fig. 3.2B). The lowest zone average was 14.9 in Powderhorn Lake in October of 2006, and the highest zone average was 33.0 in the Bay samples in February, 2006 (Fig. 3.2B). The expected salinity gradient was observed, lowest in Powderhorn Lake and highest in the bay samples.

Dissolved oxygen concentration ranged from 5.63 to 7.92 mg L^{-1} over the entire bay (Fig. 3.2C) and did not differ between sites ($p>0.05$). A difference in dissolved oxygen concentration was observed over time ($p<0.01$). Only five of the 580 sites sampled had dissolved oxygen less than 5.0 mg L^{-1}. The lowest oxygen concentration observed throughout the sampling period was 4.44 mg L^{-1} at a Near-shore site in July 2006. The lowest dissolved oxygen averages, below 6.0 mg L^{-1}, occurred in August 2005 and 2006 when temperatures peaked.

The average pH throughout the entire bay ranged from 7.59 – 8.38 (Fig. 3.2D). No difference in pH was observed between the Near-shore zone and the Bay zone ($p>0.05$), but the average pH observed in Powderhorn Lake was lower than both ($p<0.05$). Observed pH values changed over time ($p<0.05$), with the lowest pH below 8.0 occurring during the months of January, February, and April 2006.

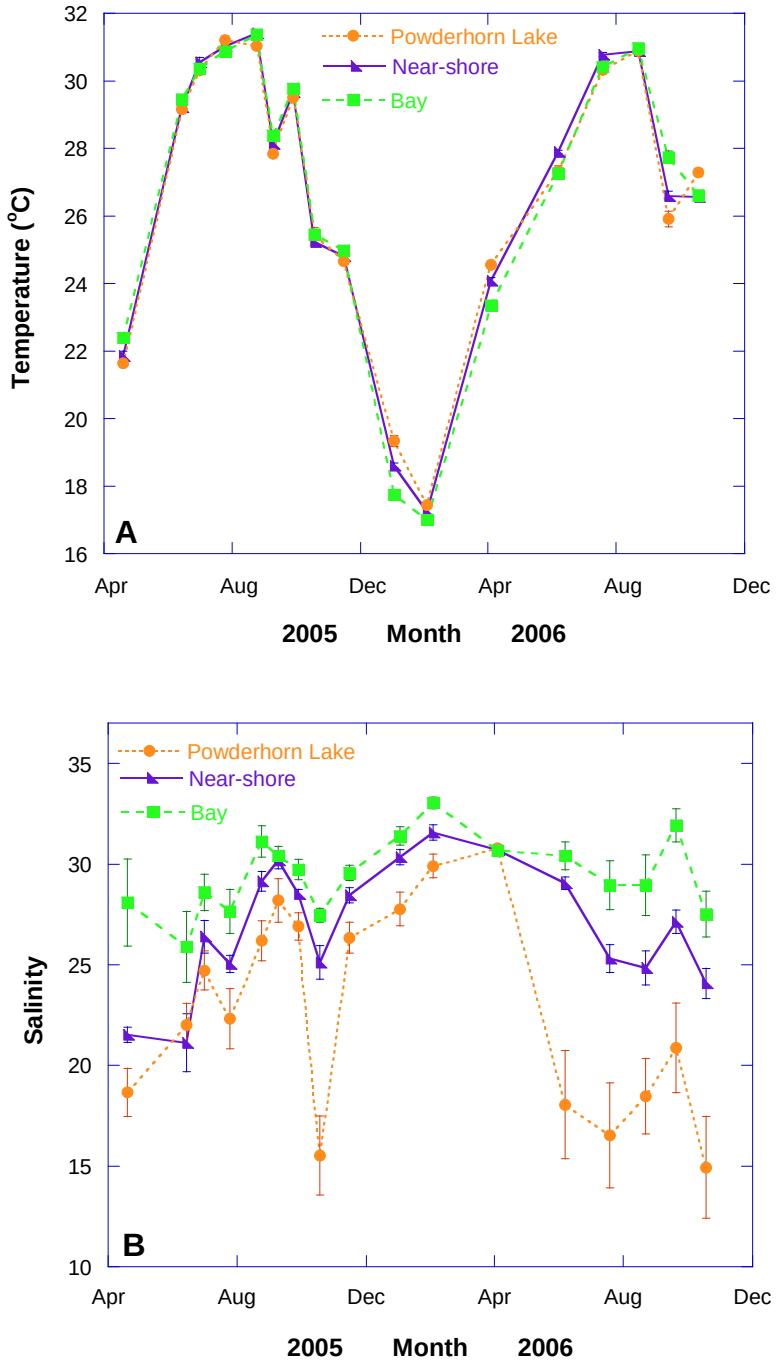


Fig. 3.2. Temporal and spatial averages of measured water parameters across sampling zones in Matagorda Bay. Parameters in the water column were averaged between all sites composing each zone (Near-shore – 8 sites, Powderhorn Lake – 5 sites, Bay – 6 sites). Surface and bottom samples were collected at sites deeper than one meter, and are included in the averages. (A) Temperature (B) Salinity (C) Dissolved oxygen concentration (D) pH. Error bars represent standard error (n=8-18).

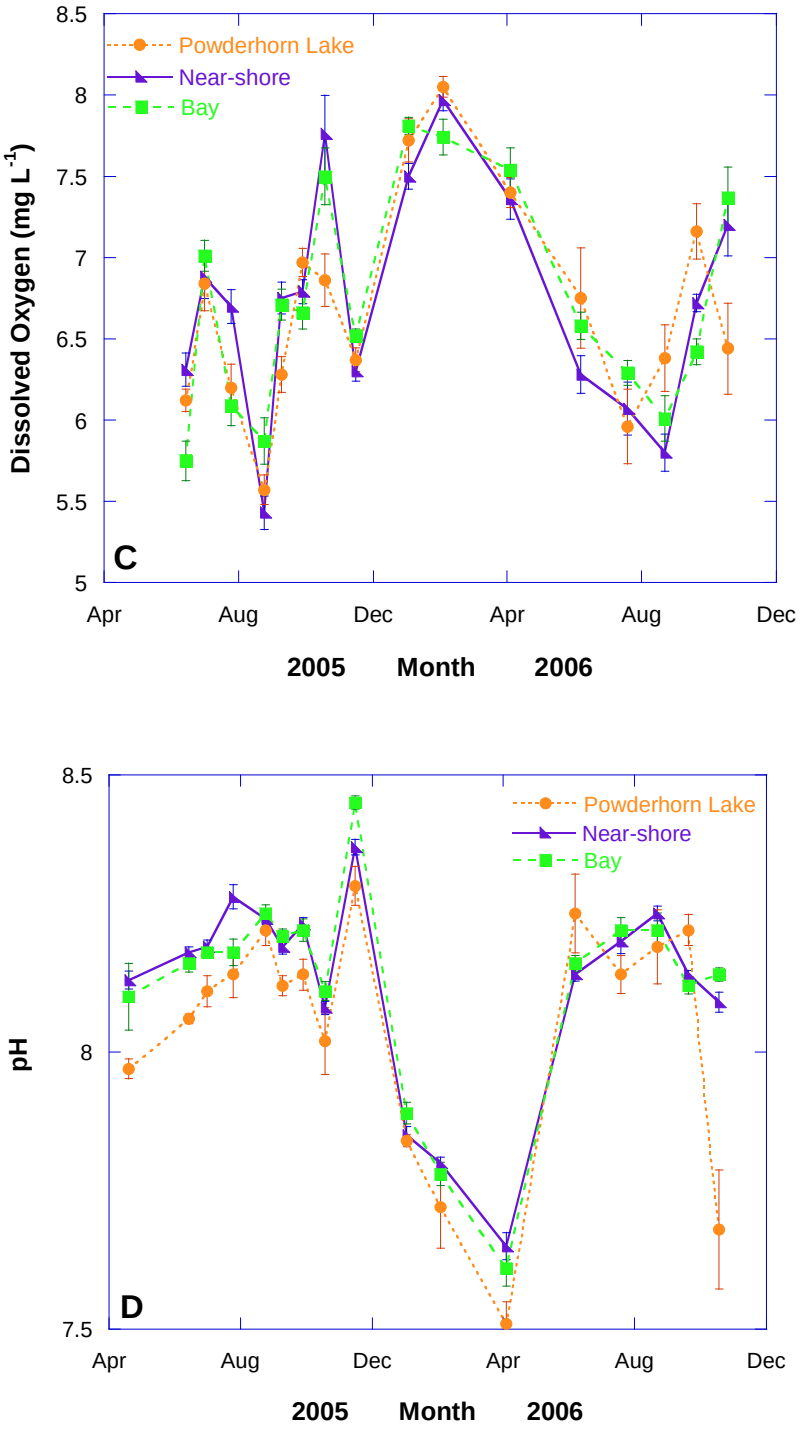


Fig. 3.2. Continued

The highest concentration of *V. vulnificus* observed at a single site was approximately $1.1x10^3$ cells mL^{-1}. The site was located within the Bay zone in late August, 2005. The second highest concentration was 520 *V. vulnificus* cells mL^{-1} from a Near-shore site July 25, 2005. The remaining positive sites for *V. vulnificus* had concentrations below 500 cells mL^{-1}. The highest mean *V. vulnificus* concentrations occurred in July and August of 2005 when water temperatures were highest (Fig. 3.3). Concentrations tapered off in September 2005, and showed only two small peaks throughout the remainder of the sampling period.

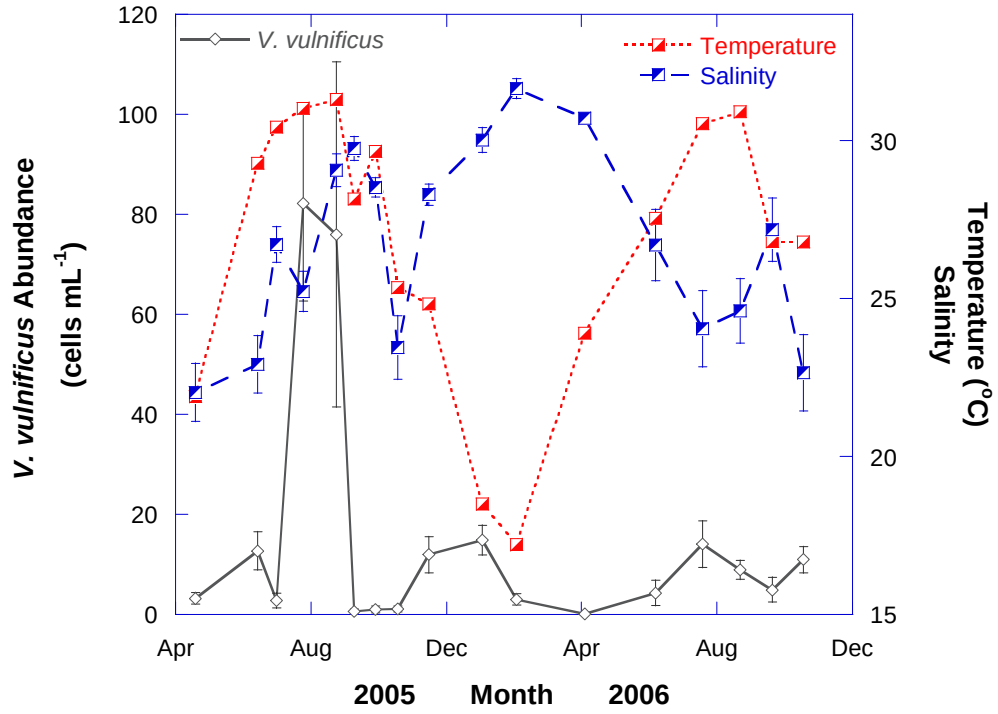


Fig. 3.3. Mean *V. vulnificus* concentrations over time against mean bay temperature and salinity. *V. vulnificus* concentrations were highest in July and August, 2005 when water temperature peaked. The highest concentration at an individual site was >1100 cells mL^{-1} in August 2005. Averages include surface and bottom samples across the sampling area. Error bars represent standard error (n=27-36).

A wide range of environmental variables were observed throughout the sampling period. The variable ranges and means for sites positive for *V. vulnificus* were similar to sites negative for *V. vulnificus* (Table 3.1). *V. vulnificus* was detected at every site at

least once throughout the sampling period. A minimum of one site was positive for *V. vulnificus* each date sampled.

Table 3.1. Comparison of environmental parameter mean values and ranges between sites that were positive and sites negative for *V. vulnificus*. The averages and ranges for environmental parameters do not differ much between the positive and negative sites.

Water parameter	Mean values for parameters measured	
	Positive sites (range)	**Negative sites (range)**
Temperature °C	26.75 (16.68-31.72)	26.9 (16.67-31.94)
Salinity	26.32 (6.52-34.95)	26.93 (2.90-35.2)
Dissolved Oxygen (mg/L)	6.63 (4.53 – 8.39)	6.77 (4.44 – 8.57)
pH	8.13 (7.65-8.51)	8.09 (7.12-8.54)
Total Bacteria	4.82E+06 (1.03E+06-1.39E+06)	4.57E+06 (7.3E+05-1.07E+07)

Analyses were performed for all 17 sampling trips as well as just for days when the water was calm. Calm days were analyzed separately to increase data quality. A higher proportion of samples collected on days when the water was rough could not be counted using epifluorescent microscopy because of high amounts of suspended material in the water column. Approximately 7% of the total samples could not be counted, 63% of which occurred on days when the water condition was rough. Approximately 12% of the total samples were not amplified with PCR targeting 16S rDNA, performed to verify successful extraction of bacterial DNA from the environment. 63% of the negative PCR samples were also from rough days.

EFFECT OF ENVIRONMENTAL PARAMETERS ON *V. VULNIFICUS* ABUNDANCE AND DISTRIBUTION

To test the hypothesis that *V. vulnificus* will out-grow other bacteria under low oxygen conditions, a simple correlation analysis was used to determine if *V. vulnificus* abundance was correlated to measured environmental physico-chemical parameters. All parameters were analyzed because a single environmental parameter does not account for total variability of a bacterium in the environment. A slight negative correlation was observed between *V. vulnificus* abundance in the water column and dissolved oxygen

concentration for total days and surface samples only (Table 3.2), indicating that *V. vulnificus* abundance increased with decreasing dissolved oxygen. Only five samples out of 580 had dissolved oxygen levels below 5.0 mg L^{-1}, and 483 samples out of 580 observed displayed dissolved oxygen levels above 6.0 mg L^{-1}. It is noted that there is a strong negative correlation ($r = -0.634$, $p < 0.01$) between dissolved oxygen concentration and water temperature that may explain the relationship between dissolved oxygen and *V. vulnificus.* The infrequent occurrence of low oxygen conditions does not allow for the hypothesis that *V. vulnificus* makes up a greater percentage of the total bacterial population under low oxygen conditions to be appropriately tested.

Correlations were also observed between *V. vulnificus* abundance and the remaining three water parameters, as well as with total bacterial abundance (Table 3.2). All correlations were significant but weak when surface and bottom samples were combined, with small coefficients of determination for each correlation (Table 3.2). The strongest correlation for any physico-chemical parameter for all samples was with temperature for calm days ($r=0.147$). Salinity had a negative correlation with *V. vulnificus* abundance, and there was a positive correlation with pH. The strongest correlation for *V. vulnificus* overall was between *V. vulnificus* concentration and total bacterial abundance for all samples on calm days ($r=0.170$).

Correlation coefficients are given for all sites including surface and bottom samples (Table 3.2). Correlations were also determined for surface and bottom samples individually for total and calm days. The significance of the correlations does change when surface and bottom samples are analyzed separately, however, significant correlations are similar and the end results are the same.

Table 3.2. Spearman's Correlation analyses for environmental parameters with *V. vulnificus* abundance and total bacterial abundance. Correlation analyses were carried out for all samples collected (top values) and samples collected on calm days only (bottom values). All sites include surface and bottom samples combined, surface sites are surface samples only and bottom sites are bottom samples only. Analyzing calm days alone eliminates 63% of the samples that could not be counted due to high amounts of suspended material, and 63% of the DNA samples that were not amplified with PCR targeting 16S rDNA.

Variable Pair		**Correlation Coefficients**		
First Element	**Second Element**	**All Sites**	**Surface Sites**	**Bottom Sites**
***V. vulnificus* cells mL^{-1}**	Dissolved Oxygen Concentration	**-0.096***	**-0.158****	-0.057
		-0.083	**-0.157***	-0.033
	Temperature	**0.100***	**0.173****	0.015
		0.147**	**0.236****	0.043
	Salinity	**-0.104***	-0.070	**-0.125***
		-0.139**	-0.111	-0.139
	pH	**0.104***	**0.139***	0.010
		0.106*	**0.147***	0.010
	Bacterial Abundance	**0.097***	0.105	0.025
		0.170**	**0.169***	**0.157***
Total Bacterial Abundance	Dissolved Oxygen Concentration	**-0.327****	**-0.315****	**-0.390****
		-0.336**	**-0.330****	**-0.398****
	Temperature	**-0.617****	**0.646****	**0.588****
		0.515**	**0.539****	**0.495****
	Salinity	**-0.420****	**-0.439****	**-0.377****
		-0.489**	**-0.474****	**-0.487****
	pH	**0.301****	**0.311****	**0.260****
		0.364**	**0.365****	**0.324****

*Significance at $p<0.05$
**Significance at $p<0.01$

Total bacterial abundance in the water column had the strongest correlation with *V. vulnificus* abundance, so the same correlation analysis was applied to total bacterial abundance. Stronger correlations were observed for total bacterial abundance than for *V. vulnificus* abundance, but the responses observed to the physico-chemical parameters were similar. The strongest correlation for total bacterial abundance was temperature (Table 3.2). Both total bacterial abundances and *V. vulnificus* abundances are expected to be greater with the higher temperatures observed in the range of this system. Total bacterial abundance was also positively correlated with pH, indicating that the higher pH observed in this system is favored by the total bacterial population. Salinity and

dissolved oxygen concentration both had negative correlations with total bacterial abundance, indicating that total abundances increase with the decreasing salinity and dissolved oxygen concentrations observed in this system.

The highest average concentrations of for both *V. vulnificus* and total bacterial occurred in late July, 2005 (82.23 cells mL^{-1}; $8.14x10^6$ cells mL^{-1}; Fig. 3.4). Abundances remained high through August 2005 (75.96 cells mL^{-1}; $6.55x10^6$ cells mL^{-1}), followed by a steep decrease in *V. vulnificus* into September. The highest average abundance of *V. vulnificus* observed through the remaining sampling season was in January, 2006 (14.85 cells mL^{-1}). Total bacterial abundance peaked again in June 2006 ($5.65x10^6$ cells mL^{-1}), and July 2006 was the final small peak for *V. vulnificus* abundance (14.11 cells mL^{-1}).

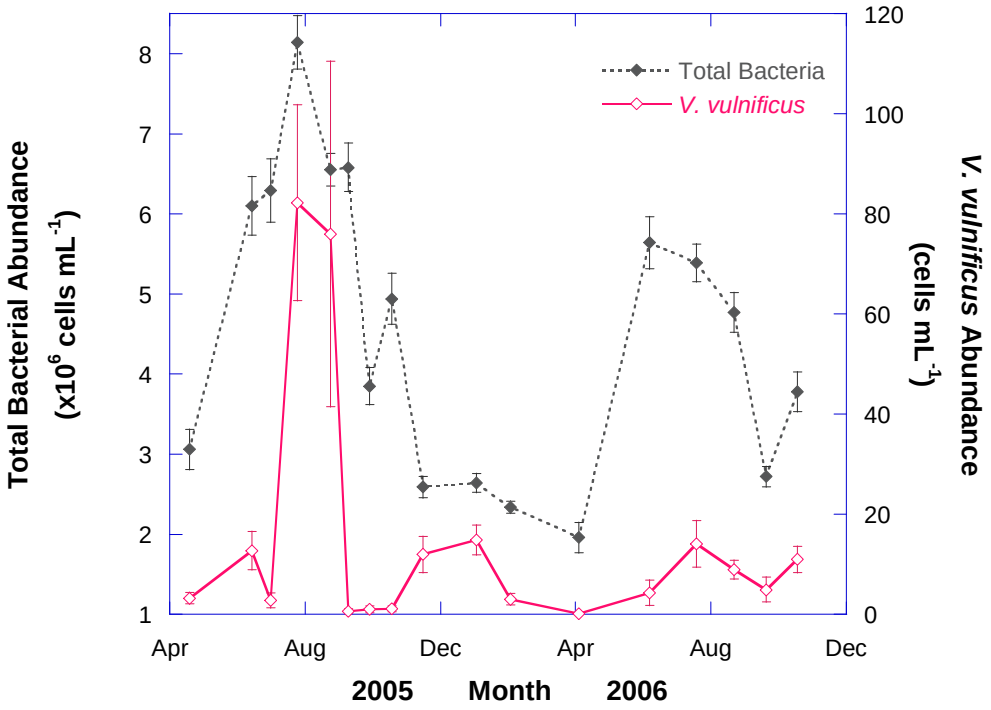


Fig. 3.4. *V. vulnificus* and total bacterial abundances over time. Total bacterial and *V. vulnificus* average abundances were calculated for all sites in Matagorda Bay each date sampled from April 2005 to October 2006. Averages include surface and bottom samples. The strongest correlation for *V. vulnificus* abundance was with total bacterial abundance in the water column on calm days (r=0.195). The highest average *V. vulnificus* concentration was 82.23 cells mL^{-1} in late July, 2005. Error bars indicate standard error (n=27-36).

Individual parameters do not account for all variability of a bacterium in the environment. Interactions may make one factor outside of an optimal range more tolerable. A Multivariate Analysis of Variance (MANOVA) was applied to determine significant interactions between environmental variables and *V. vulnificus* and total bacterial abundance. Variances between the groups tested were not homogenous; therefore the significance level was set at a p-value of ≤ 0.01. On calm days, the only significant contributions to *V. vulnificus* variation were sampling date and an interaction of sampling date and location ($p<0.01$; $p<0.01$). No interactions between the environmental parameters measured were significant for the variation of *V. vulnificus* abundance in Matagorda Bay. Date and an interaction between date and location were also significant for the variation of total bacterial abundance on calm days ($p<0.01$, $p<0.01$). Salinity and an interaction between salinity and pH also contributed to total bacterial variability in Matagorda Bay on calm days ($p<0.01$, $p<0.01$).

A step-wise multiple regression was carried out using both total bacterial abundance and *V. vulnificus* abundance to determine which parameters, if any, could be used for prediction of abundance. Of the four parameters examined, temperature, salinity, dissolved oxygen concentration, and pH, only temperature was used in the model for *V. vulnificus* abundance. The model was weak, accounting for less than 2% of the total variation in *V. vulnificus* abundance (data not shown).

The regression model for total bacterial abundance included temperature and salinity for total days and calm days. For total days, temperature accounts for 33% of the variability. Salinity contributes an additional 8%, while dissolved oxygen only contributes to 1% of the variability, accounting for a total of 42% of the variability for total bacterial abundance. The equation derived from the linear regression for total days is $(\text{Total Bacterial Abundance}_{total}) = -3.57\text{x}10^{6} + (3.33\text{x}10^{5} * \text{Temperature}) - (1.24\text{x}10^{5} * \text{Salinity}) + (3.87\ \text{x}10^{5} * \text{Dissolved Oxygen Concentration})$. On calm days, temperature accounted for 27% of the variability, salinity added 12%, and pH contributed a final 2% to the total variability of total bacterial abundance for a total of 41%. The resulting equation is: $(\text{Total Bacterial Abundance}_{calm}) = -1.00\ \text{x}10^{7} + (2.14$

$x10^5$*Temp) – (1.48 $x10^5$*Salinity) + (1.98 $x10^6$*pH). These results are consistent with temperature having the strongest correlations with bacterial abundance in both cases.

SPATIAL VARIABILITY OF *V. VULNIFICUS* IN MATAGORDA BAY OVER THREE ZONES

To test the hypothesis that Powderhorn Lake serves as a point source for *V. vulnificus*, the nineteen sites sampled within Matagorda Bay were grouped into three zones; Powderhorn Lake, Near-shore, and Bay (Fig. 3.1). The Powderhorn Lake zone is composed of five sites within the lake. The Near-shore zone has eight relatively shallow sites near the lake mouth and close to the shoreline of the bay, including one site at a marina in Port O'Connor, TX. The Bay zone includes six relatively deep sites slightly further off-shore. Zones were determined based on the expected salinity gradients to be observed from the lake to the bay. Seventeen dates were analyzed for total samples, and 12 dates were analyzed for calm day samples.

V. vulnificus abundance in the water column varied over time (Fig. 3.5). The Bay zone had a high average *V. vulnificus* concentration in August 2005, skewed by the single site with the highest concentration of over 1100 *V. vulnificus* cells ml^{-1}. The frequency of detection of *V. vulnificus* in the three zones is displayed in the Table 3.3 below. For all 17 days analyzed, there was not a large difference in the frequency of detection (Table 3.3). No significant differences between sites with respect to frequency of detection or *V. vulnificus* abundance were observed ($p>0.05$, $p>0.05$). Bacterial abundance, however, was significantly different between zones ($p<0.01$).

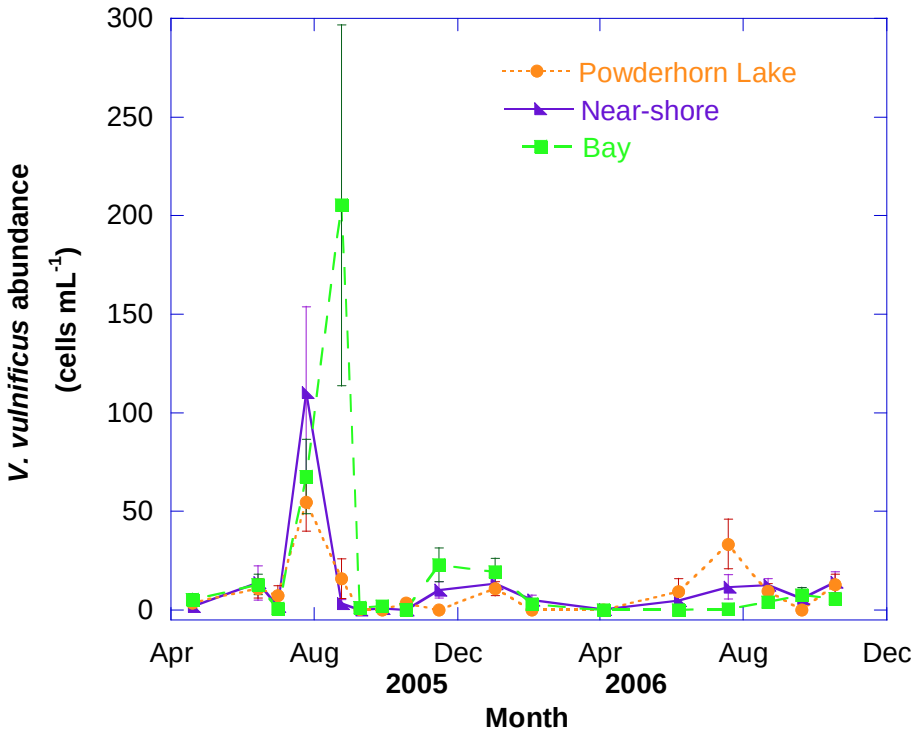


Fig. 3.5. Mean *V. vulnificus* concentrations over three zones in Matagorda Bay. *V. vulnificus* abundance varied over time but the trend was similar between zones. Average values include surface and bottom samples. Error bars represent standard error (n=8-12).

A higher percentage of samples from Powderhorn Lake were positive for *V. vulnificus* than either of the other two zones on calm days (Table 3.3). The frequency of *V. vulnificus* detection between zones on calm days appears to be significant, however, due to high variance between samples, there is not a significant difference in detection frequency between zones ($p>0.05$). No difference was observed in *V. vulnificus* abundance between zones ($p>0.05$), but there was a significant difference in total bacterial abundance ($p<0.01$).

Rough days showed a much lower frequency for *V. vulnificus* detection than calm days. There is a significant difference between the frequency of detection and *V. vulnificus* abundance between calm days vs. rough ($p<0.01$, $p<0.01$). While significant, it must be kept in mind that a majority of the samples that could not be counted or were not successfully amplified with PCR targeting 16S rDNA were from days when the

water was rough. It is possible that the difference is due to low sample quality caused by water condition.

Table 3.3. Frequency of *V. vulnificus* in three Matagorda Bay zones under different water conditions. Different water conditions were used to determine if mixing of water on rough days had an affect on abundance and distribution patterns. Rough day results may be biased due to a higher proportion of low-quality samples. Analyzing calm days alone eliminates 63% of the samples that could not be counted due to high amounts of suspended material, and 63% of the DNA samples that were not amplified using PCR targeting 16S rDNA.

Water Condition	Zone	Total Samples	% of Samples Positive for *V. vulnificus*	Total Bacteria cells mL^{-1}
All	Lake	153	35%	5.54E+06
	Near-shore	229	32%	4.61E+06
	Bay	198	37%	4.05E+06
	Total	580	34%	4.65E+06
Calm	Lake	108	44%	5.81E+06
	Near-shore	163	34%	5.00E+06
	Bay	144	36%	4.34E+06
	Total	415	38%	4.98E+06
Rough	Lake	45	13%	4.32E+06
	Near-shore	66	27%	3.53E+06
	Bay	54	41%	3.27+06
	Total	165	28%	3.61E+06

While there was not a difference in *V. vulnificus* abundance and detection frequency between zones, it was detected more frequently and in higher abundances from surface samples than bottom samples for total days sampled ($p<0.05$, $p<0.05$; Table 3.4). Only *V. vulnificus* abundance was significantly different between surface and bottom samples ($p<0.05$) on calm days. Examining surface samples and bottom samples individually across zones made no difference in the outcome of distribution patterns. *V. vulnificus* abundance in surface samples across zones showed no difference for total or calm days ($p>0.05$; $p>0.05$). There was also no difference across zones with bottom samples for total or calm days ($p>0.05$; $p>0.05$).

Table 3.4. Frequency and concentrations of *V. vulnificus* in surface and bottom water column samples. Sites with only one sample taken were considered surface samples. There was a statistically significant difference in *V. vulnificus* abundance between surface and bottom samples for total and calm days ($p<0.05$).

Water Condition	Depth	Total Samples	% of Samples Positive for *V. vulnificus*	Average *V. vulnificus* cells mL^{-1}	Total Bacteria cells mL^{-1}
Total	Surface	317	38%	18.32	4.85E+06
	Bottom	263	31%	11.78	4.40E+06
Calm	Surface	226	41%	23.35	5.19E+06
	Bottom	189	33%	14.73	4.72E+06
Rough	Surface	91	32%	5.33	3.70E+06
	Bottom	74	26%	4.24	3.49E+06

SPATIAL VARIABILITY OF *V. VULNIFICUS* IN MATAGORDA BAY WITH RESPECT TO OYSTER BEDS

Oyster beds were identified within the sampling sites with the help of a fishing map and input from a local fisherman. One site with oyster beds was in the Powderhorn Lake zone, three were within the Near-shore zone, and two were in the Bay zone (Fig. 3.6). Sites with oyster beds were compared to the remaining 13 sites without oyster beds to determine if there was a significant difference in the concentrations and frequency of detection of *V. vulnificus* in the water column near oyster beds.

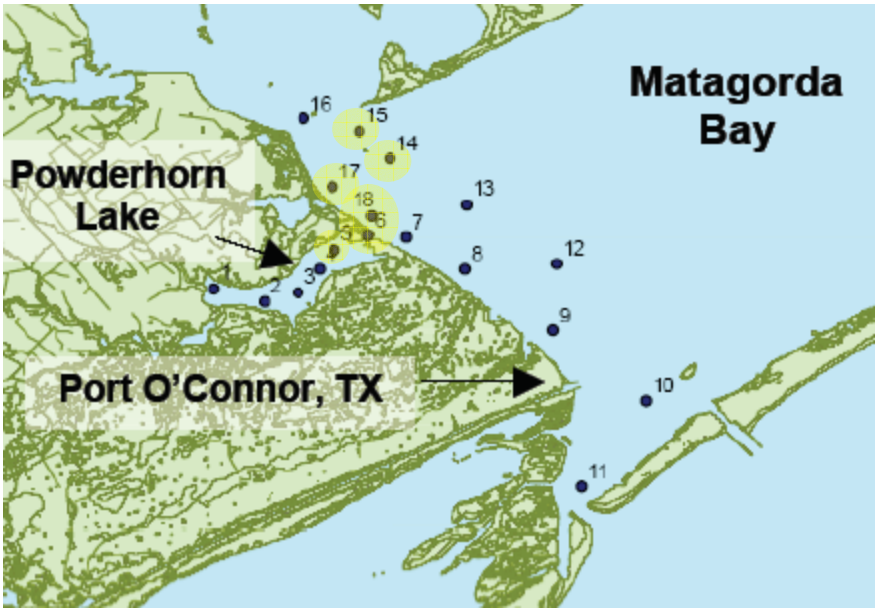


Fig. 3.6. Sampling sites with and without oyster beds in Matagorda Bay. Sites near oyster beds are highlighted with yellow. Oyster bed sites are distributed in all three zones; one within the Powderhorn Lake zone, three within the Near-shore zone, and two within the Bay zone.

A lower percentage of samples near oyster beds were positive for *V. vulnificus* for both total and calm days sampled (Table 3.5). There was no significant difference in *V. vulnificus* detection between sites with oyster beds present and those without for total and calm days ($p>0.05$, $p>0.05$). No difference was observed in *V. vulnificus* concentration between sites with oyster beds and those without for total days and calm days ($p>0.05$, $p>0.05$; Fig. 3.7). There was no significant difference in detection and abundance of *V. vulnificus* in sites with oyster beds and those without, therefore, we cannot conclude that higher concentrations of *V. vulnificus* exist in the water column where oyster beds are present.

Table 3.5. Frequency of *V. vulnificus* in sites with oyster beds present and sites where oyster beds are absent. Six sites with oyster beds were compared to 13 sites without oyster beds. There is not a significant difference in detection or *V. vulnificus* abundance in sites with oyster beds and those without.

Water Condition	Oyster Reef	Total Samples	% of Samples Positive for Vv	Total Bacteria cells mL^{-1}
Total	Present	202	32%	4.69E+06
	Absent	378	36%	4.63E+06
Calm	Present	144	34%	5.08E+06
	Absent	271	39%	4.93E+06
Rough	Present	58	28%	3.60E+06
	Absent	107	29%	3.62E+06

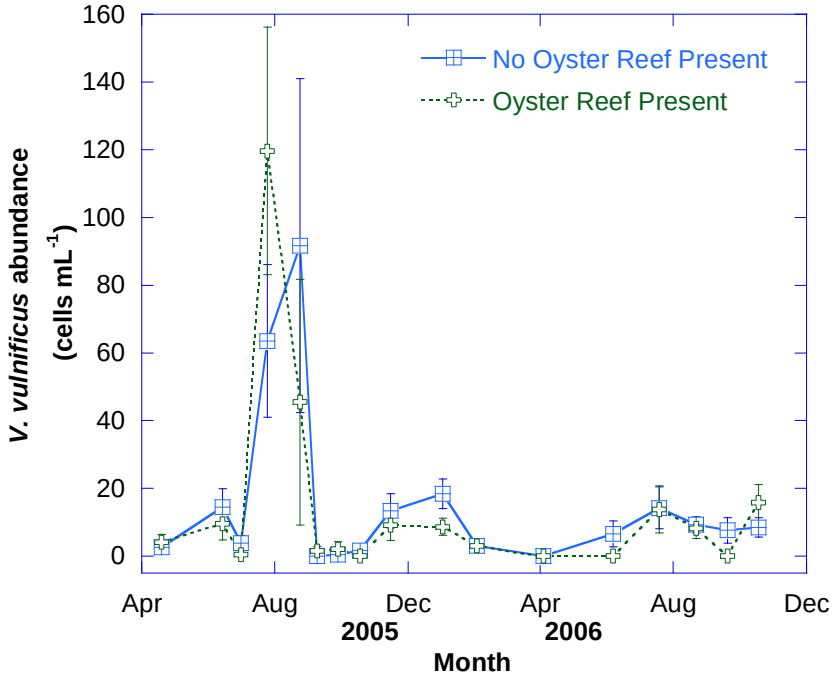


Fig. 3.7. Mean *V. vulnificus* concentrations at sites with oyster beds and sites without. *V. vulnificus* abundance varied over time but the trend was similar. Average values include surface and bottom samples. Error bars represent standard error (n=12-24).

PATCHY DISTRIBUTION OF *V. VULNIFICUS* IN MATAGORDA BAY

V. vulnificus abundance and distribution was patchy throughout the sample region of Matagorda Bay and over time. Every site sampled was positive for *V. vulnificus* at least once during this study, and every date sampled had at least one site that was positive for *V. vulnificus* (Fig. 3.8a-q). The dates with the highest average *V. vulnificus* abundances were in the summer of 2005, July 25 (Fig. 3.8d) and August 24 (Fig. 3.8e). The next highest averages, highest to lowest, were seen in January 2006 (Fig. 3.8j), July 2006 (Fig. 3.8n), and November 2005 (Fig. 3.8i). Detection frequencies over 50%, listed highest to lowest, were on the sampling dates January 2006 (Fig.3.8j), July 25, 2005 (Fig. 3.8d), August 24, 2005 (Fig. 3.8e), and October 2006 (Fig. 3.8h).

a. Rough

Fig. 3.8. Distribution of *V. vulnificus* in Matagorda Bay. The values indicate ranges of *V. vulnificus* concentrations in the water column. Blue dots represent sites without *V. vulnificus* detection. 1=<10 cells mL^{-1}; 2=11-100 cells mL^{-1}; 3=101-500 cells mL^{-1}; 4= >500 cells mL^{-1}. Numbers alone represent surface samples, underlined numbers are bottom samples. Some values may be high due to non-specific binding as well as specific binding in the Q-PCR reaction. Calm or rough water conditions are marked below on each individual map. Figures 8a-8q are in chronological order.

b. calm

c. calm

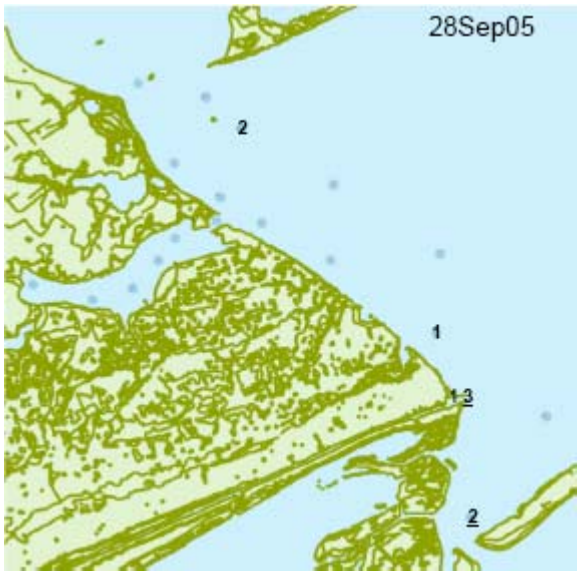
Fig. 3.8. Continued

d. calm

e. calm

Fig. 3.8. Continued

f. rough



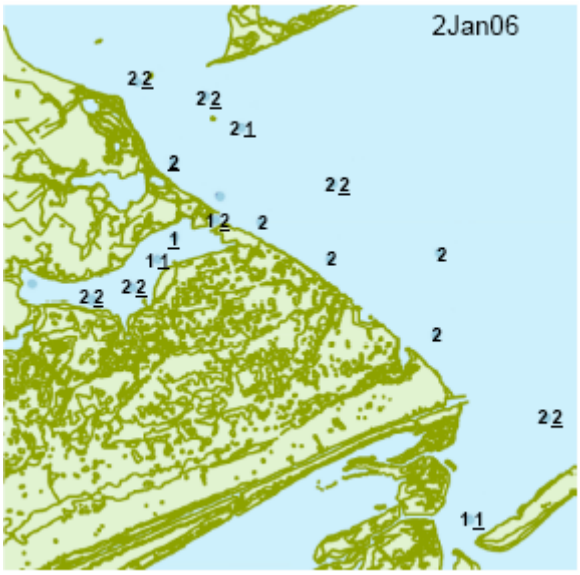
g. calm

Fig. 3.8. Continued

h. calm

i. rough

Fig. 3.8. Continued



j. calm

k. rough

Fig. 3.8. Continued

l. calm

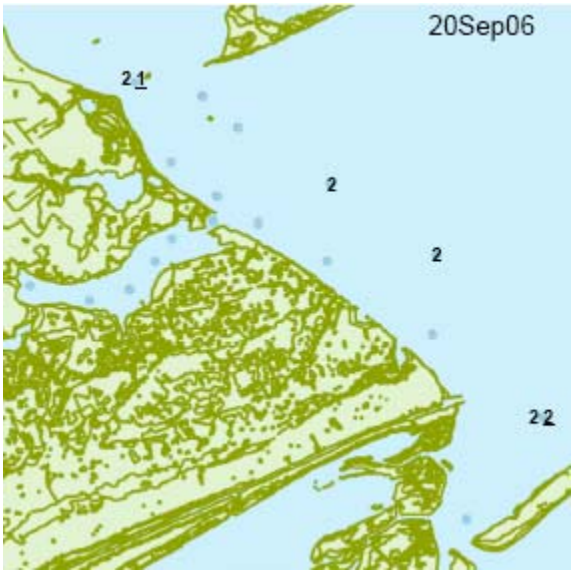
m. calm

Fig. 3.8. Continued

n. calm

o. calm

Fig. 3.8. Continued



p. rough

q. calm

Fig. 3.8. Continued

Patchiness in *V. vulnificus* was observed spatially and temporally. Sites in close proximity varied in concentration, sometimes observing a site with high concentrations of *V. vulnificus* and no detection at a site close by. *V. vulnificus* was also frequently detected at a surface site but not at the corresponding bottom site, even when the column depth was less than two meters. The patchiness in distribution makes it difficult to identify a point source for *V. vulnificus* in the water column.

Detection was discontinuous over time, as two sampling dates with high detection frequencies flanked sample dates with low detection frequencies. *V. vulnificus* was detected $\geq$ 50% of the time at only three of the 36 different sampling sites. *V. vulnificus* was detected at a minimum of one site each sampling trip throughout the entire sampling period, and over a wide range of temperatures and salinities. Concentrations varied between sites and dates in which the measured environmental parameters were similar, displaying a patchiness over environmental conditions. The wide range of parameters in which *V. vulnificus* was detected, and the lack of consistency under similar conditions makes it difficult to predict abundance and distribution patterns based on the environmental parameters measured.

CHAPTER IV
DISCUSSION AND CONCLUSIONS

INTRODUCTION

Vibrio vulnificus has been isolated from temperate and tropical climates across the globe, and are commonly found in Gulf Coast and East Coast estuaries (Kelly, 1982; Tamplin et al., 1982; Wright et al., 1996; Hoi et al., 1998; Motes et al., 1998; Hervio-Heath et al., 2002; Lin et al., 2003; Pfeffer et al., 2003; Fukushima and Seki, 2004) in the United States. Many studies have been conducted to determine significant environmental parameters affecting the abundance and distribution of *V. vulnificus* in the environment. It has been well established that temperature and salinity both play important roles in the detection and concentrations of *V. vulnificus* in the water column (Kelly, 1982; Kaspar and Tamplin, 1993), and that the dominant parameter often depends on geographic location (Wright et al., 1996; Lipp et al., 2001; Pfeffer et al., 2003).

No studies have been reported to date on *V. vulnificus* in Matagorda Bay, Texas. This study aimed to evaluate spatial and temporal variation of *V. vulnificus* abundance in Matagorda Bay because of the potential for physico-chemical gradients that may contribute to abundance and distribution patterns. Understanding the dynamics of *V. vulnificus* abundance in Matagorda Bay will contribute to the overall knowledge of the geographic distribution of *V. vulnificus*.

ENVIRONMENTAL PARAMETERS CONTRIBUTING TO *V. VULNIFICUS* ABUNDANCE AND DISTRIBUTION

Several experimental and environmental studies have been carried out to determine optimal temperature and salinity ranges for *V. vulnificus*. Laboratory based studies have found that *V. vulnificus* will tolerate higher salinities longer at lower temperatures (Kaspar and Tamplin, 1993). The optimal salinity range for experimental studies was roughly 5-20 and temperature ranges between 13-22°C and 37°C (Kelly,

1982; Kaspar and Tamplin, 1993). In the environment optimal temperature ranges have usually been from 15 to >30°C and salinities below 15 (Kelly, 1982; Tamplin et al., 1982; Oliver et al., 1983; Kaysner et al., 1987; Lipp et al., 2001; Pfeffer et al., 2003).

Temperatures in Matagorda Bay remained within the optimal range the entire sampling period. Salinity was outside of the optimum range for most of the study, but *V. vulnificus* was detected over a broad range of salinities. Other studies have isolated *V. vulnificus* from such broad salinity ranges, but the spatial scales of those studies were much greater (Oliver et al., 1983; Kaysner et al., 1987). Salinity and temperature have been well studied, but it is recognized that there are other factors that contribute to the variability of *V. vulnificus* in the water column that have yet to be so well established.

The first main objective of this study was to determine if dissolved oxygen concentration in the water column contributed to the abundance and distribution of *V. vulnificus*, a facultative anaerobe. Corpus Christi Bay, a South Texas bay near Matagorda Bay, experiences seasonal hypoxia in the summertime, attributed to high bottom water salinities and water column stratification (Ritter and Montagna, 1999). Water column stratification contributes to hypoxia formation by preventing oxygen-rich surface water from mixing with bottom water, resulting in low oxygen bottom water conditions (Diaz, 2001). Powderhorn Lake was expected to reach low oxygen levels as it was the most likely region in Matagorda Bay to exhibit stratification, and water within the lake was said to become stagnant in the summer.

Stratification was only observed during three of the seventeen sampling events and was primarily limited to Powderhorn Lake. There were high amounts of precipitation in 2004 (Fig. 4.1). Several Vibrio infections were reported in the area in July 2004, following a month of heavy rainfall in June. Precipitation was much lower in 2005 than 2004, and increased in 2006 but still did not reach the levels of 2004 (Fig. 4.1). The low occurrence of stratification could be explained by few precipitation events for freshwater inputs to the bay system over the two summers sampled.

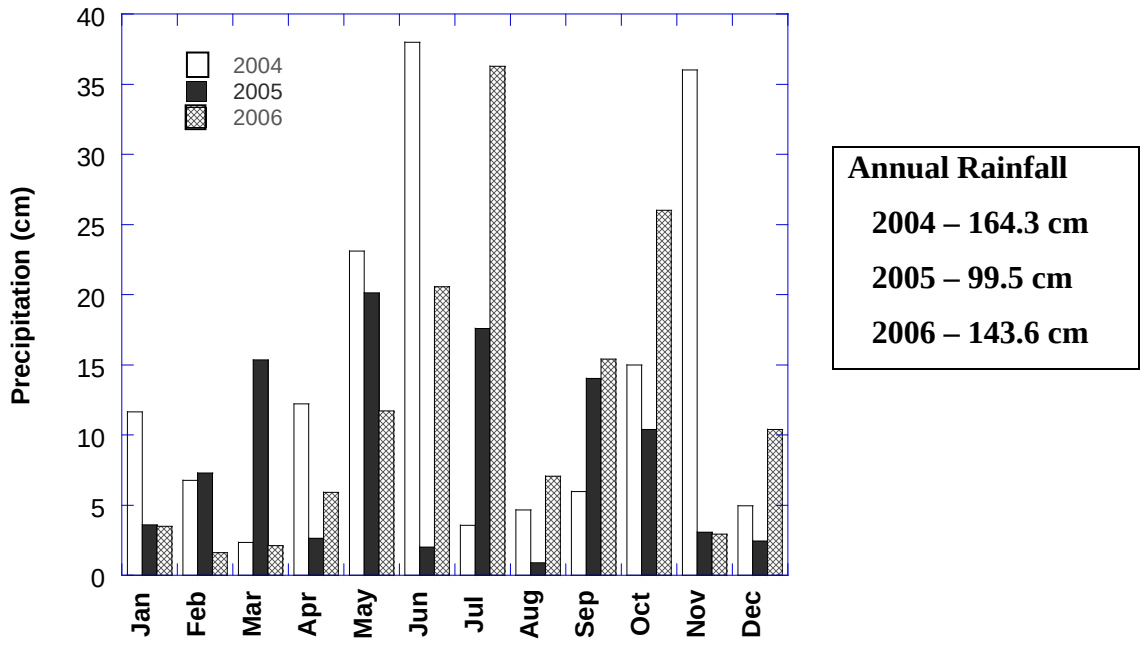


Fig. 4.1. Average monthly rainfall for 2004, 2005, and 2006. The highest annual rainfall was in 2004. 2005 was dry compared to 2004 and 2006, and 2006 did not have as much precipitation as 2004. Data from Lower Colorado River Association - River Operation Center.

Dissolved oxygen concentrations were rarely low enough to stress aerobic bacteria in the environment throughout this study. Matagorda Bay is shallow and well-mixed, and with the low amounts of rainfall did not exhibit stratification often. The well mixed nature of the bay did not allow for the development of hypoxic conditions. The bay was observed to be a well oxygenated system throughout the sampling period, making it difficult to establish whether or not *V. vulnificus* will outgrow other bacteria under low oxygen conditions when the oxygen levels are rarely low enough to stress aerobic organisms.

Dissolved oxygen has not been extensively studied or reported as a significant factor in *V. vulnificus* abundance in the water column. The relationship between *V. vulnificus* and dissolved oxygen concentration was evaluated in this study because, like with temperature and salinity, the importance of a relationship with dissolved oxygen may be dependent on geographic location. Dissolved oxygen was negatively correlated to *V. vulnificus* abundance in the water column, similar to one other study (Pfeffer et al.,

2003). This is most likely due to lower dissolved oxygen concentrations in the water column as a result of higher temperatures, as *V. vulnificus* is more prevalent in warmer months.

Consistent with other studies, a positive correlation was seen between *V. vulnificus* abundance and temperature in the water column (Lipp et al., 2001; Lin et al., 2003; Pfeffer et al., 2003). The correlation in this study is not as strong as other studies, possibly because the temperature range was never outside of the optimum range established for *V. vulnificus.* Pfeffer et al. (2003) found temperature to account for 47% of *V. vulnificus* variability, with no correlation to salinity. On the contrary, Lipp et al. (2001) found that salinity was more important than temperature in the prediction of *V. vulnificus* abundance.

Salinity had a weak but significant negative correlation with *V. vulnificus.* A similar relationship was found by Lin et al. (2003), attributed to the range of salinity (5-25) observed in Galveston Bay, TX. Since temperature remained close to the ideal range throughout the study period, it was thought that salinity might have a stronger relationship with *V. vulnificus* than temperature. The salinity range in Matagorda Bay was greater than that for Lin et al. (2003), but *V. vulnificus* was detected across the range, possibly accounting for the weak correlation.

Matagorda Bay experienced variation in pH over time, with bay averages ranging from 7.58-8.38. Lipp et al. (2001) found a significant relationship between *V. vulnificus* in sediments and pH, but did not identify such a relationship within the water column. This study saw a slight positive correlation between *V. vulnificus* and pH in the water column. Estuaries typically have a pH of 8.2, but can experience gradients due to riverine input of more acidic waters. A gradient was observed between zones in Matagorda Bay. Powderhorn Lake had a lower pH than the other two zones throughout most of the study. pH variations have not been well studied in regards to *V. vulnificus* in the water column, and could potentially contribute to predictive models. Given that *V. vulnificus* is found in estuaries, where pH gradients are not uncommon, more refined studies for pH are needed.

V. vulnificus abundance in the water column is due to an interaction of physico-chemical parameters. Lower temperatures increase tolerance of *V. vulnificus* to unfavorable salinities (Kaspar and Tamplin, 1993). Seasonal environmental studies have shown the highest concentrations when temperatures are warm and salinities are low (Kelly, 1982; Lipp et al., 2001). Interactions between the environmental parameters for this study were tested, but none significantly contributed to *V. vulnificus* abundance. This result was expected because of the weak correlations observed between the environmental parameters and *V. vulnificus* abundance.

Models created by other studies accounted for 47% of the variation in the water column with temperature alone (Pfeffer et al., 2003), and up to 49% using temperature and salinity (Lipp et al., 2001). No such model could be established for this study. When attempted, only temperature was included, and temperature alone contributed to only 2% of the variability of *V. vulnificus* in Matagorda Bay. The weak regression is most likely due to the temperature remaining within the optimal range for most of the study, and the wide range of salinities from which *V. vulnificus* was detected.

The hypothesis that *V. vulnificus* out-grows other bacteria in a low oxygen environment could not be appropriately tested due to few occasions where dissolved oxygen may have been low enough to stress aerobes in the environment. The correlation seen between *V. vulnificus* and dissolved oxygen concentration in this system was more likely due to the strong negative correlation between temperature and dissolved oxygen. While there were significant correlations between *V. vulnificus* abundance in the water column and the four variables tested, no significant interactions could be determined. Some of the relationships were consistent with previous studies, but there was not a clear dominating factor as observed in past studies.

Relationship of *V. vulnificus* Abundance with Total Bacterial Abundance

The strongest correlation observed in all samples for *V. vulnificus* was with total bacterial abundance on calm days throughout the sampling period. The correlation between *V. vulnificus* and total bacterial abundance was stronger than any of the

physico-chemical parameters measured on calm days. The averages of total bacterial abundance and *V. vulnificus* abundance followed a similar trend throughout the sampling period. The highest averages for both occurred in July and August of 2005, and decreased into the winter months.

The similar trends observed corresponded with the similar responses of the total bacterial population with the environmental parameters. Total bacterial abundances were higher during the warmer months, and also had a tendency to be higher in lower salinities. A similar response was also seen with dissolved oxygen concentrations. A negative correlation with dissolved oxygen concentration was anticipated with *V. vulnificus* as it is a facultative anaerobe, but this relationship was not expected for total bacterial abundance.

If *V. vulnificus* makes up a greater percentage of the total bacterial population in low oxygen environments, it was expected that total bacterial abundance would have a positive correlation with dissolved oxygen concentration. The total bacterial concentration is much greater than that of *V. vulnificus* alone. Although *V. vulnificus* might increase compared to the remaining bacterial population, an increase of the single species would not be enough to increase the entire population. The remaining bacterial population would be expected to decrease under low oxygen conditions, thus the total population would decrease. The negative correlation with the total bacterial population could be explained in part by the strong negative correlation between dissolved oxygen concentration and water temperature, and temperature having the strongest correlation to total bacterial and *V. vulnificus* abundance.

Vibrios have been found to make up a high percentage of the culturable bacterial population. Oliver et al. (1983) found that Vibrios make up 26-40% of the bacterial population averaging $9.6x10^3$ cells mL^{-1}. Other studies report *V. vulnificus* composing 0.4-17% of total culturable bacteria (Chan et al., 1986; Wright et al., 1996), or 7.7% of culturable *Vibrio* spp. (Pfeffer et al., 2003). Direct microscopy counts of cells concentrated onto membrane filters give total bacterial abundances more than two orders of magnitude higher than culturing methods (Jannasch and Jones, 1959). The above

percentages are likely to be high, as the average total bacterial abundance estimate from culturing is more than two orders of magnitude less than the average total bacterial abundance in estuaries (Ducklow, 2000). Enumeration of cells using fluorescent oligonucleotide direct-counting found that *V. vulnificus* made up 0.02-0.3% of the total bacterial population (Heidelberg et al., 2002). While these studies reported the percentage of the total bacterial population attributed to *V. vulnificus*, none reported a similar relationship to that observed in this study between *V. vulnificus* and total bacterial abundance.

DISTRIBUTION OF *V. VULNIFICUS* THROUGHOUT THREE MATAGORDA BAY ZONES

The second objective of this study was to determine if Powderhorn Lake serves as a point-source for *V. vulnificus*. It was expected that Powderhorn Lake would exhibit the lowest salinities, and therefore harbor the highest concentrations of *V. vulnificus.* Previous studies have found significant differences between different groups of sampling stations (Lipp et al., 2001), and a trend of decreasing *V. vulnificus* concentrations with increasing salinities from an upper bay to the lower bay (Wright et al., 1996).

The Matagorda Bay sampling sites were grouped into three zones based on the anticipated salinity gradient. Zones displayed the expected gradient in average salinity, with the lowest in Powderhorn Lake, then Near-shore, and the highest salinities in the Bay zone. There was not a significant difference in *V. vulnificus* concentrations between zones as hypothesized, regardless of water condition or depth in the water column. The sites displaying the highest average *V. vulnificus* concentrations were actually located in the Bay and Near-shore zones. Powderhorn Lake exhibited a lower pH than the rest of the bay, and there was a positive correlation between *V. vulnificus* and pH which was stronger than the negative correlation with salinity in surface samples. This could partially explain why concentrations were not higher in Powderhorn Lake, but the relationship with pH needs to be more extensively studied.

Wind driven water movement could affect the distribution of *V. vulnificus*. It is possible that *V. vulnificus* proliferates in Powderhorn Lake and is wind driven into the bay. However, if *V. vulnificus* is flushed from Powderhorn Lake into the rest of the bay, it would likely still be detected in Powderhorn Lake when it was detected in the other two Matagorda Bay zones. This was not always the case, but it is noted that many low quality samples were from Powderhorn Lake. Most low quality samples were from windy days when the water was choppy, likely due to high amounts of suspended material collected with the samples. Low quality samples could contribute to the lack of *V. vulnificus* detection in Powderhorn Lake when it was detected in Near-shore and Bay zone sites. While it is possible these physical conditions influence the distribution patterns for *V. vulnificus,* data for wind and currents were not collected throughout this study.

V. vulnificus was not detected more frequently or in higher abundances in Powderhorn Lake than the other two zones. Even though Powderhorn Lake consistently exhibited the lowest salinities, salinities were most often outside of the optimal salinity range and the relationship between *V. vulnificus* and salinity was weak. Since neither higher frequencies nor concentrations of *V. vulnificus* were observed in Powderhorn Lake, it was concluded that Powderhorn Lake does not serve as a point-source for *V. vulnificus.*

DISTRIBUTION OF *V. VULNIFICUS* WITH RESPECT TO OYSTER BEDS

The third objective for this study was to determine if the presence of oyster beds could be used as an indicator of higher *V. vulnificus* concentrations in the water column. Many studies have been conducted on *V. vulnificus* abundances in oysters and the water column, but they have not focused on a difference in abundance in regards water surrounding oyster beds and water without oyster beds (Tamplin et al., 1982; Oliver et al., 1983; Kaysner et al., 1987; Lin et al., 2003; Lin and Schwarz, 2003).

Wright et al. (1996) observed that *V. vulnificus* was more likely to be isolated from sediments that contained oysters or were surrounding oyster beds than sediments

that did not. It was expected that similar findings occur in the water column near oyster beds as oysters concentrate microbes from the water column, and may also release concentrated microbes back into the water column. When the six sites near oyster beds in this study were compared to the remaining thirteen sites without oyster beds, no significant differences were observed in *V. vulnificus* abundance or total bacterial abundance in the water column.

The exact proximity of water samples in Matagorda Bay to oyster beds are not known, so it is difficult to determine how representative these samples are for being near oyster beds. The discrepancy between *V. vulnificus* in the water column compared to sediment samples may be due to dispersion of bacteria in the water column. If *V. vulnificus* is released from oysters embedded in the sediments, the bacteria might remain in the sediments and never reach the water column. It is also possible that bacteria that are released into the water column can become attached to particles and sink back out of the water column into the sediments (Davies et al., 1995).

Higher bacterial abundances are found in sediment pore waters than in the water column (Schmidt et al., 1998). Lipp et al. (2001) found higher concentrations of *V. vulnificus* in the sediments than in the surrounding water, but could not establish a relationship between concentration in the sediments and the water column. The positive correlation between bacterial abundance and *V. vulnificus* abundance in the water column may apply to sediments as well, however, we did not analyze sediments in this study.

Multiple factors contribute to the abundance and distribution of microorganisms in the water column, and oyster beds as a biotic factor cannot alone contribute to the variations in the concentrations of *V. vulnificus* found in the water column. *V. fischeri* have a symbiotic association with squid. A recent study examined concentrations of *V. fischeri* in the water column in which squid were present (Jones et al., 2007). Higher abundances of *V. fischeri* were found in the water column where squid were present near Hawaii, but the same was not true for squid near Australia (Jones et al., 2007), showing that the presence of a biotic source is not the only determining factor for the prevalence

of a microorganism. Biotic factors and physico-chemical parameters are important influences on microbial distributions, but cannot be considered completely independent of one another.

V. vulnificus was not detected in higher concentrations at sites near oyster beds. While the difference between sites with and without oyster beds was insignificant, the relationship between *V. vulnificus* in the environment surrounding oysters is an interesting avenue to pursue. Neither sediments nor oysters were examined in this study. It may be useful to sample sediments, oysters and water from the same site, as well as sediments and water without oysters present to better understand the dynamics of *V. vulnificus* in the environment surrounding oysters. This is necessary to determine if oysters do directly contribute to *V. vulnificus* abundance in sediments and water immediately surrounding oysters.

PATCHY DISTRIBUTION OF *V. VULNIFICUS* IN MATAGORDA BAY

The spatial distribution of *V. vulnificus* in Matagorda Bay did not display a predictable pattern. The best description of the spatial and temporal distribution is that it was patchy. While microorganisms were once thought to be homogenously distributed throughout environmental systems, patchy distribution is found even at the microliter scale in seawater (Long and Azam, 2001). Patchiness of *V. vulnificus* has previously been observed on a scale similar to that of this study (Heidelberg et al., 2002), but is not frequently reported for *V. vulnificus* in the environment. This may be because most of the previous studies have sampled fewer sites within a larger spatial scale, or sampled less frequently over time (Kelly, 1982; Tamplin et al., 1982; Oliver et al., 1983; Lipp et al., 2001; Lin et al., 2003; Pfeffer et al., 2003).

This study collected surface and bottom samples from 19 sites over a small spatial scale, while previous studies had less intense sampling schemes. Pfeffer et al. (2003) collected all of their samples from six sites located near rivers, and so only lower salinity waters were sampled for *V. vulnificus.* Lipp et al. (2001) collected surface samples at 12 sites monthly for one year, yielding a high detection frequency. Wright et

al. (1996) only had eight sites in Chesapeake Bay, four close to a fresh water source, and four away from the source. None of these studies reported patchy distribution of *V. vulnificus*. In this study, distribution was patchy between sites as well as between surface and bottom samples. If previous studies had been carried out with more intensive sampling schemes, it is possible that the same patchiness in distribution could have been observed.

The highest concentrations of *V. vulnificus* in this study were observed in late July and August of 2005. Levels did not reach the same concentrations again, even though sampling continued throughout an additional summer with similar temperatures and greater amounts of precipitation. Year to year variation in *V. vulnificus* concentrations has been observed in oysters, with levels in oyster tissue increasing by more than an order of magnitude from one year to the next (Jackson et al., 1997). Tamplin et al. (1982) noticed fluctuations between *V. vulnificus* abundance in the water column between sampling trips, but samples were only collected six times over 18 months. The same fluctuations may have still been observed with a higher temporal resolution sampling regime.

Frequency of *V. vulnificus* detection is variable between various environmental studies. The percentage of samples positive for *V. vulnificus* in the water column with culturing methods ranges from 5.9% in samples from the west coast (Kaysner et al., 1987) to 45% in Texas (Lin et al., 2003) to 95% in Florida samples (Lipp et al., 2001). Since culturing techniques are constraining due to the amount of non-culturable bacteria in the environment, it might be expected that direct detection methods would yield a higher frequency of detection. In this study, *V. vulnificus* was detected in only 34% of total water samples analyzed, and concentrations were similar to those in studies using culturing methods. Two samples from this study had *V. vulnificus* abundances greater than 500 cells mL^{-1}, but most samples were between 10-100 cells mL^{-1}. The highest abundance of *V. vulnificus* found by Lipp et al. (2001) was 190 CFU mL^{-1} of seawater, with a mean of 12.1 CFU mL^{-1}. Wright et al. (1996) isolated between 40-600 cells mL^{-1} of seawater.

Distribution patterns previously have shown *V. vulnificus* to follow salinity gradients, or occur at higher frequency and abundance in the lower salinity sites for their studies (Kelly, 1982; Wright et al., 1996). This was not the case in Matagorda Bay. The highest concentrations were found in sites when the temperature was high, but salinity was high compared to previous reports. Studies that found *V. vulnificus* concentration and distribution was not correlated to salinity sampled stations with salinities remaining in the optimal range (Pfeffer et al., 2003). *V. vulnificus* detection and concentrations varied between sites with similar environmental parameters, including those sites which had salinities and temperatures within the optimal range.

The observed spatial patchiness makes it difficult to identify a point-source for *V. vulnificus* in Matagorda Bay. The observed temporal patchiness contributes to the weak correlations with the measured parameters as the most significant changes occurred with time. The weak predictors observed in this study suggest that there are other potential contributors to *V. vulnificus* abundance and distribution that were not measured in this study. It is possible that nutrients play a role in the distribution, as nutrients are not always homogenously distributed in the environment (Long and Azam, 2001). There are many potential factors affecting *V. vulnificus* distribution in the environment that should be considered further.

Studies for *V. vulnificus* dynamics in estuaries can improve in several ways. Sampling schemes should focus on spatially and temporally intensive sampling patterns for improved analysis of distribution patterns throughout various estuarine systems. Additional environmental parameters should be considered, as the primary factors measured in this study did not contribute to reliable prediction for *V. vulnificus* in the water column. Biotic factors also influence bacteria in the water column, and should be considered in conjunction with abiotic factors for their contribution to *V. vulnificus* variability. Intensive sampling and consideration of more potential influences on *V. vulnificus* in the environment may increase understanding of the patchy distribution observed in this system, and also enhance predictive modeling systems in the future.

METHODS

Traditional methods for detection and enumerating *V. vulnificus* in the environment have been culture dependent (Kelly, 1982; Oliver et al., 1983; Lipp et al., 2001; Lin et al., 2003). Several studies have had difficulty isolating *V. vulnificus* from environmental samples, oyster or water, when temperatures fell below 15°C (Oliver et al., 1995; Pfeffer et al., 2003). Less than 1% of environmental bacteria are culturable (Tsai and Rochelle, 2001), eliminating a large fraction of the total bacterial population when relying on culture techniques alone. To overcome the constraints of non-culturable bacteria, this study used extraction of nucleic acids from environmental samples followed by Q-PCR.

The densities of *V. vulnificus* in this study were similar to those found by Lipp et al. (2001), even with the difference in methods (culture dependent vs. culture independent). Detection frequency, however, was lower in Matagorda Bay than previous studies in Gulf Coast estuaries using culture methods (Lipp et al., 2001; Lin et al., 2003). The lower detection frequency in this study may be attributed to more intensive sampling and the higher salinities observed in Matagorda Bay than the other bay systems.

The DNA extraction protocol used was chosen for its high DNA yield in small water samples (Boström et al., 2004) optimized for use in Q-PCR. The extraction method was modified slightly due to fluorescence of the coprecipitant used by the author in DNA quantitation. The reliable extraction efficiency in the protocol was not obtained in this study. The determination of efficiency for this environmental study was attempted by calculating the expected amount of DNA from total bacterial counts and comparing it to the DNA yield. However, due to other sources of DNA in the environment such as protozoa and phytoplankton, the efficiency could not be accurately determined.

Boström et al. (2004) were able to obtain high yields of DNA with samples from 2- 400 mL of seawater, but indicated that sample volume may influence DNA recovery. In this study, between 50 and 200 mL of bay water was filtered for each sample

throughout the collection period. DNA extraction efficiency may have increased with smaller water samples, as Boström et al. (2004) yielded higher amounts of DNA per mL^{-1} of seawater when filtering smaller volumes, however, dissolved DNA could contribute to the greater recovery due to unknown adsorption to the filters.

Q-PCR has been used as an effective tool for detecting and quantifying pathogens (He et al., 2002; Okubara et al., 2004). Panicker et al. (2004) used SYBR-Green I chemistry to optimize detection of *V. vulnificus* in oysters and seawater. Detection sensitivity for *V. vulnificus* in unseeded seawater was determined at 100 *V. vulnificus* cells mL^{-1} of seawater (Panicker et al., 2004). In this system, *V. vulnificus* could confidently be quantified when 300 genome copies were in 10 ng of environmental template; equivalent to approximately 100 cells mL^{-1} of filtered bay water. Detection below this limit is reliable, but concentration values may not be.

Some estimations of *V. vulnificus* abundance could be high due to the contribution of non-specific binding to fluorescence lowering the C_T value. Estimations of total *V. vulnificus* in the water-column could be low due to the method in which *V. vulnificus* abundance was calculated. Concentrations were calculated using the ratio of *V. vulnificus* copies to total bacterial copies in the 10 ng of template DNA used for the Q-PCR reaction. Since the DNA was extracted from environmental samples, the DNA from sources other than bacteria was also likely extracted as well as bacterial DNA, making the percentage of *V. vulnificus* in the total bacterial population low. The calculated abundances were used for statistical analyses, but values were binned into four levels for visualization of distribution throughout the bay over time.

Calculations for concentrations of *V. vulnificus* in the water column could be improved by developing a universal primer set targeting a gene that has only one copy per bacterial cell. This would allow total bacterial abundance to be quantified in the same manner as *V. vulnificus*. A ratio can then be established for total bacteria and *V. vulnificus* in a given amount of DNA. The ratio can be applied to direct epifluorescent counts of bacteria in the water column, and eliminate the need to use the amount of template DNA for the calculations of bacterial abundance in the water column.

Optimizing total bacterial quantitation using Q-PCR would help establish more reliable ratios for abundance calculations if DNA extraction efficiency is questionable.

FUTURE DIRECTIONS

While it is well established that temperature and salinity play a large role in the abundance and distribution of *V. vulnificus*, there are less well studied factors that contribute to the variability of *V. vulnificus* in the environment. This study attempted to explore the possibility that dissolved oxygen concentration plays a significant role in *V. vulnificus* ecology, but conditions did not allow this to be thoroughly tested. An unexpected positive correlation with pH suggests that it could be significant for predictive models in some areas, but needs to be explored further.

There are also other possible variables that were not considered in this study. Nutrients or utilizable forms of iron in the environment could contribute to *V. vulnificus* variability. Microscale patchiness has been observed, attributed to the patchiness of the availability of nutrients in the environment (Long and Azam, 2001). It is possible that nutrient distribution could be a contributor to the patchy distribution of *V. vulnificus* on a much larger scale.

Biotic influences may also contribute to *V. vulnificus* variability in the water column, and can be taken to an even smaller scale than oysters. Bacterial interactions can affect what species are present in close proximity to one another. Symbiotic interactions may attract bacteria to each other, while antagonistic interactions may drive others away. It would be interesting to examine the bacterial community structure and compare between sites where *V. vulnificus* was detected and sites where it was not. It is not likely that community structures will remain the same, as the total community can be expected to exhibit the same patchiness that was seen with *V. vulnificus.* However, it is possible that there are a few species that are ubiquitous across sites positive for *V. vulnificus* and sites where *V. vulnificus* was undetectable, opening up questions for the types of interactions that may be taking place.

There was a positive correlation between total bacterial abundance and *V. vulnificus* in this study. The relationship between total bacterial abundance and the contribution of *V. vulnificus* to the total population potentially shows the same trend. This relationship could be examined, and samples in which *V. vulnificus* makes up a greater percentage may indicate when *V. vulnificus* successfully competes against other bacteria in response to changes in the environmental system. Community structure analysis may show changes in the dominant bacteria, and identify other species that are able to compete for nutrients. *V. vulnificus* was detected in relatively low numbers through most of the study, so it is not expected that it would be a dominant player in the bacterial community. Examining bacterial community structure would be a new, molecular biological-based approach to examine relationships between *V. vulnificus* and other naturally occurring bacteria in the water column.

Temperature is well established as being an important determinant in *V. vulnificus* abundance in the water column and in oysters. Temperature had the strongest correlation with *V. vulnificus* out of all four environmental parameters analyzed. With rising global temperatures, the time in which estuarine temperatures remain within the optimal range increases, as well as the zone in which temperatures may be in the optimal range for pathogen proliferation (Harvell et al., 2002). The temperature in Matagorda Bay remained high enough for *V. vulnificus* proliferation over an 18 month sampling period. When temperature is ideal but distribution is patchy, other factors must be playing a major role in the abundance and distribution. The increased time and space for optimal temperatures increases the importance of establishing other significant predictors for *V. vulnificus* concentrations and distribution in the environment because temperature alone becomes a less reliable predictor.

To accomplish developing better predictive systems, exploration of *V. vulnificus* ecology must continue to go beyond temperature and salinity, and in higher resolution. As well established factors are not always reliable indicators of when and where *V. vulnificus* is present in high concentrations, it is important continue exploring other possible indicators. Examining biotic factors as well as other physico-chemical

parameters in different environmental systems may contribute to the understanding of *V. vulnificus* ecology and allow for the improvement of predictive systems. Strong, reliable prediction systems are important for stakeholders of the natural resources that bays and estuaries provide.

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APPENDIX

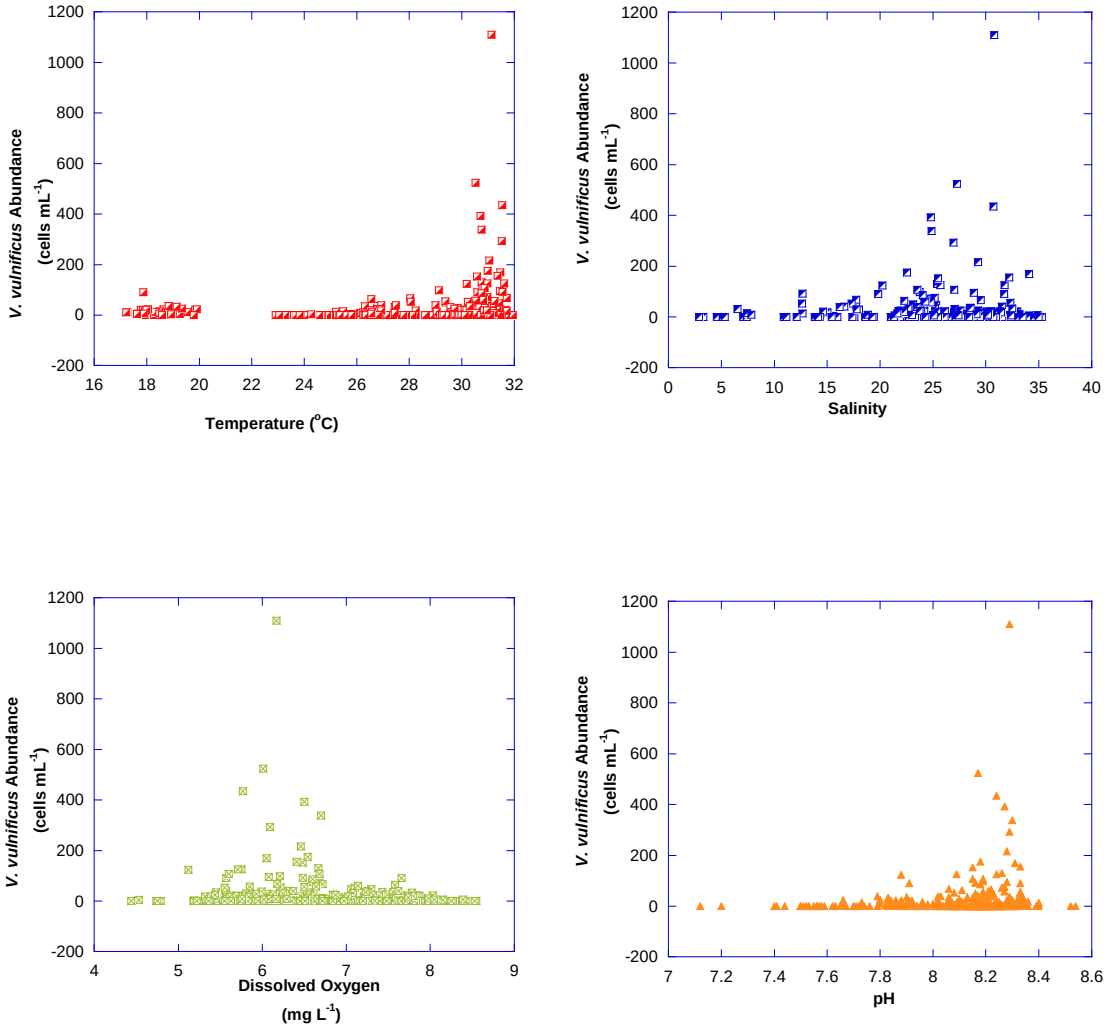

Fig. A.1. *V. vulnificus* abundance vs. each measured parameter on calm days only. Each graph includes surface and bottom samples.

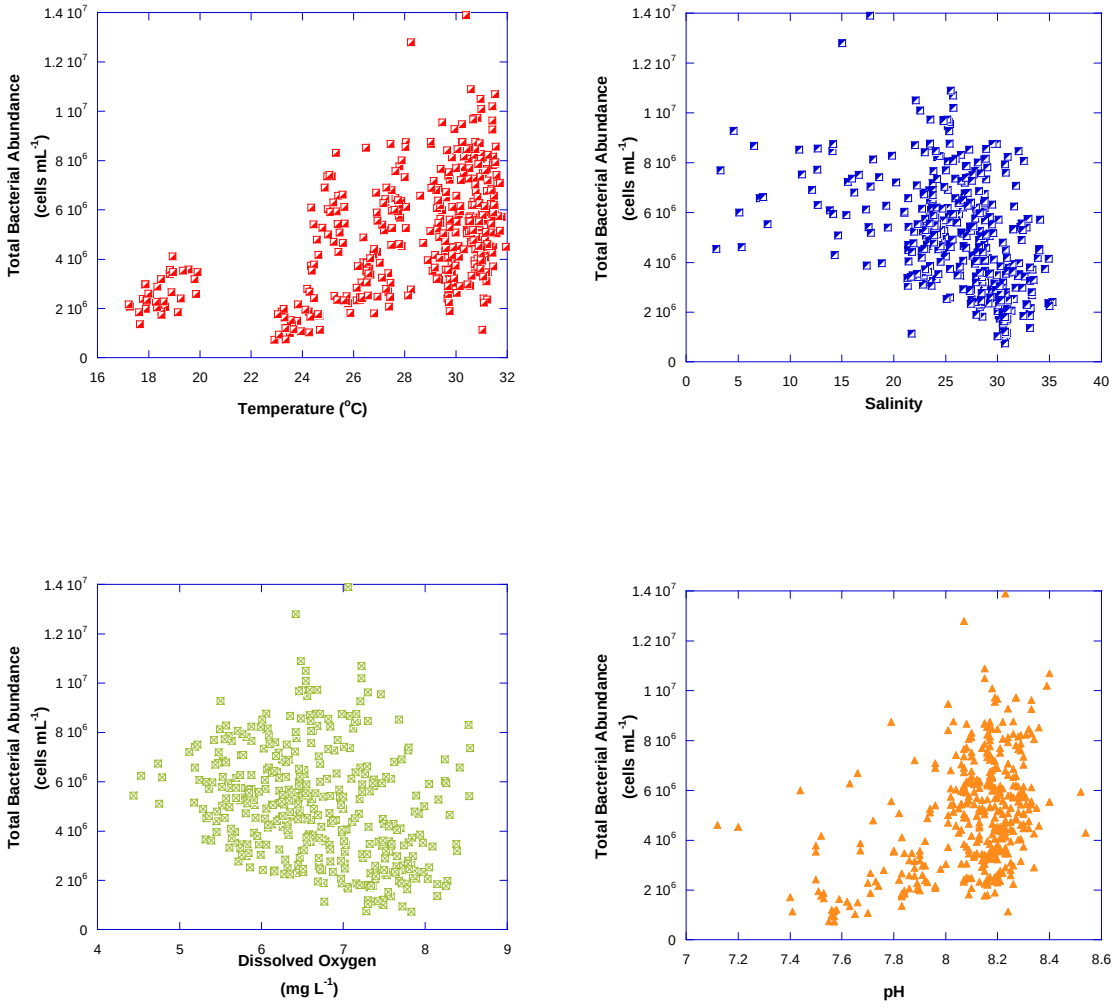

Fig. A.2. Total Bacterial abundance vs. each measured parameter on calm days only. Each graph includes surface and bottom samples.

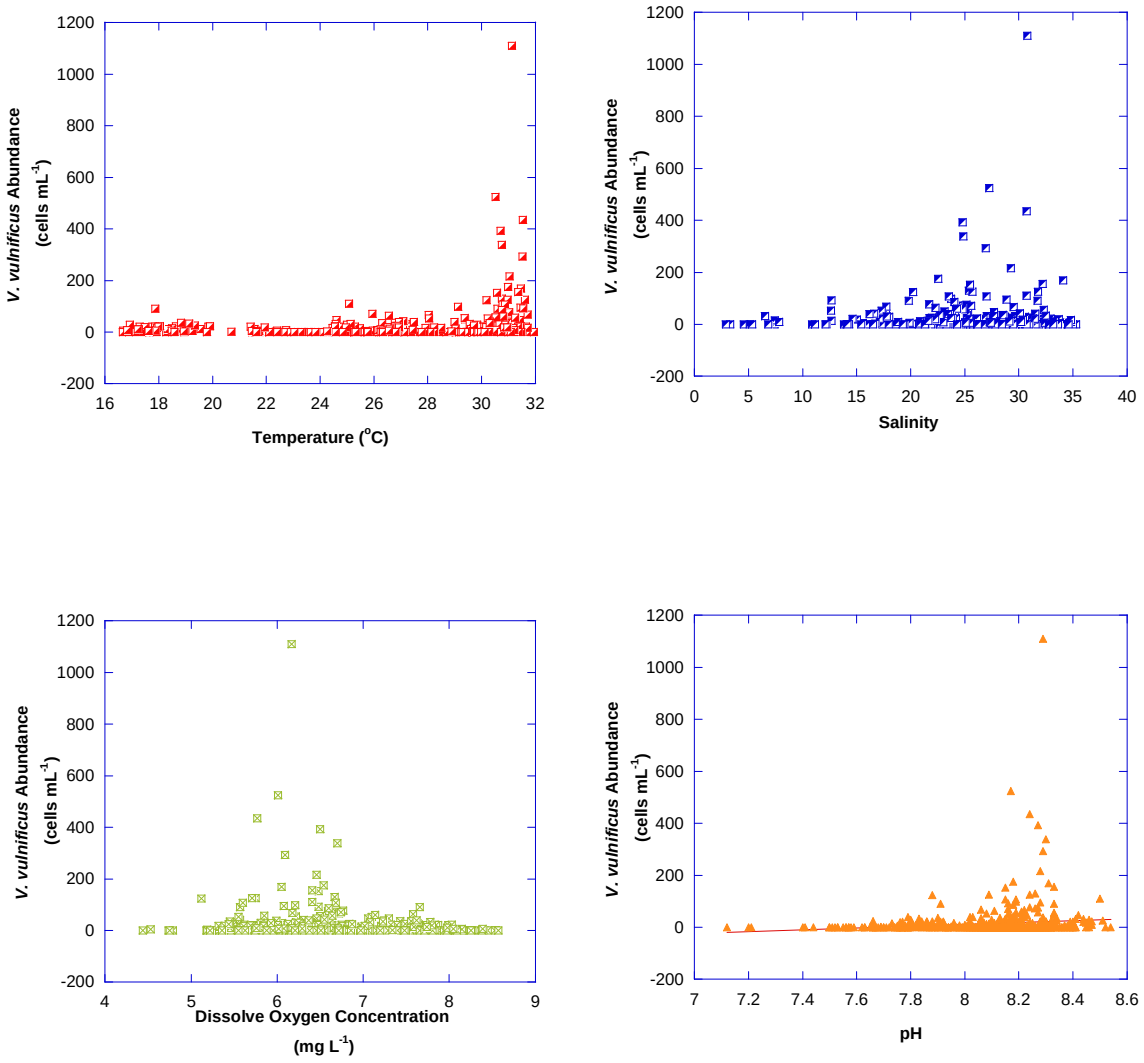

Fig. A.3. *V. vulnificus* abundance vs. each measured parameter on all days sampled. Each graph includes surface and bottom samples.

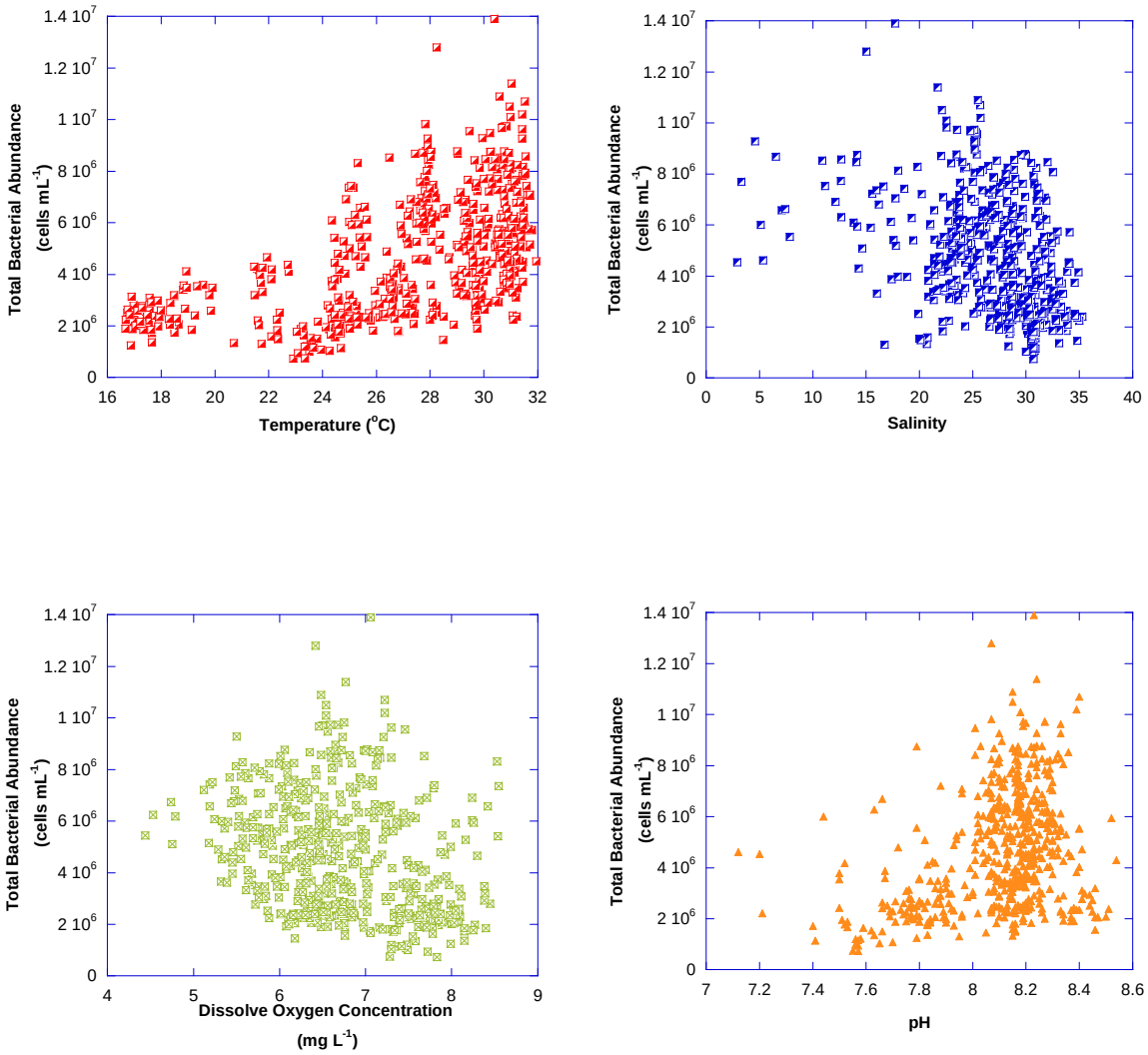

Fig. A.4. Total Bacterial abundance vs. each measured parameter on all days sampled. Each graph includes surface and bottom samples.

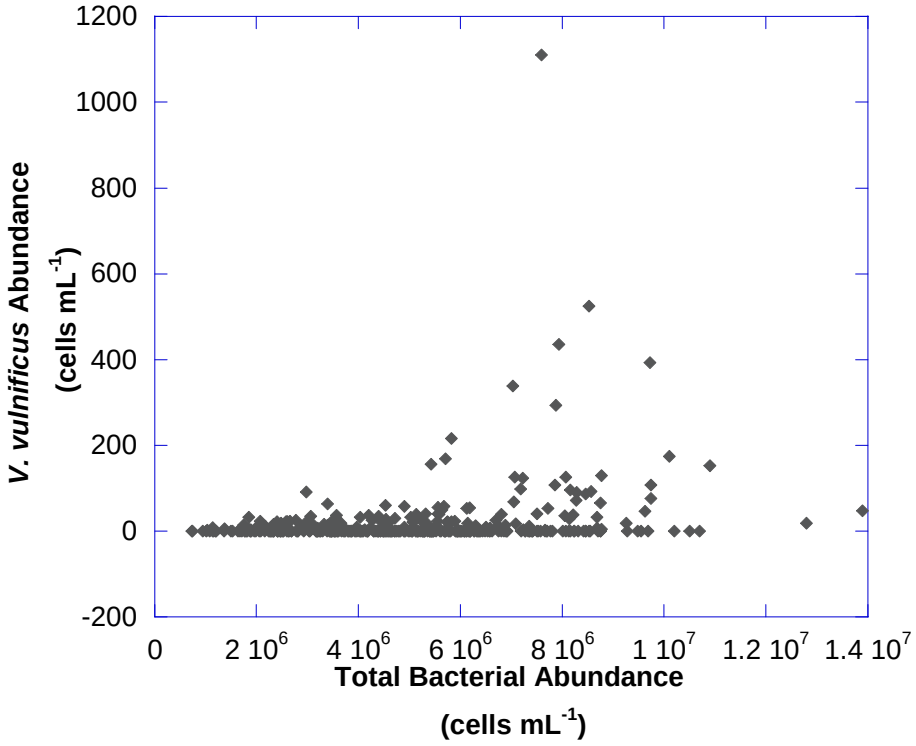

Fig. A.5. *V. vulnificus* Abundance vs. Total Bacterial abundance for all days sampled. Surface and bottom samples are included.

Table A.1. Binned *V. vulnificus* values in Matagorda Bay. Green = both duplicates had only specific binding. Blue = both duplicates had specific and non-specific binding. Pink = variation between duplicates. 1=<10 *V. vulnificus* cells mL^{-1}, 2= 10-100, 3=100-500, 4=>500.

Zone	Site	04/19/05	06/14/05	07/01/05	07/25/05	08/24/05
Near Shore	LM/A-S		2			1
	LM/A-B	X	X			X
	LM/B-S	1			2	1
	LM/B-B	1			2	1
	LM/C-S					
	LM/C-B	2	2		3	
	LM/CD-S				2	
	LM/CD-B				3	
	LM/D-S	1	1		3	1
	LM/D-B				3	
	LM/E-S			2		1
	LM/E-B	1		1		
	CASE		2		4	
	CLKS-S	X	X			2
	CLKS-B	X	X		2	2
Powderhorn Lake	PL/A-S	2			2	
	PL/A-B		1	1	2	
	PL/B-S	1	2		3	
	PL/B-B					
	PL/C-S		1		2	
	PL/C-B	1				1
	PL/D-S		2	2	2	2
	PL/D-B	1	1		2	2
	PL/E-S		2	2	2	2
	PL/E-B	X	X	X	X	X
Bay	SO/01-S		1		2	3
	SO/01-B	2			2	1
	SO/02-S	X	2		3	4
	SO/02-B	X				
	SO/03-S	X	1			3
	SO/03-B	X			3	3
	SO/04-S	X	1		3	1
	SO/04-B	X			2	1
	SO/05-S	1	2		2	2
	SO/05-B		2			3
	SO/06-S	1	2		3	3
	SO/06-B			1	2	1
Sites + for *V. vulnificus*		12	17	6	25	22

Table A.1. Continued.

Zone	Site	09/09/05	09/28/05	10/18/05	11/15/05	01/02/06
Near Shore	LM/A-S				2	2
	LM/A-B	X		X	X	X
	LM/B-S					2
	LM/B-B					X
	LM/C-S					1
	LM/C-B					2
	LM/CD-S					
	LM/CD-B					
	LM/D-S				2	
	LM/D-B				2	2
	LM/E-S				2	2
	LM/E-B					2
	CASE		1		2	2
	CLKS-S		1			
	CLKS-B	1	3			X
Powderhorn Lake	PL/A-S	1				
	PL/A-B					1
	PL/B-S					1
	PL/B-B			1		1
	PL/C-S			2		2
	PL/C-B					2
	PL/D-S					2
	PL/D-B					2
	PL/E-S			2		
	PL/E-B	X	X	X	X	X
Bay	SO/01-S				2	2
	SO/01-B			1	3	2
	SO/02-S				1	2
	SO/02-B					
	SO/03-S				2	2
	SO/03-B				2	2
	SO/04-S				1	2
	SO/04-B				1	2
	SO/05-S	2	2			2
	SO/05-B				2	1
	SO/06-S				2	1
	SO/06-B		2		2	1
Sites + for *V. vulnificus*		3	5	4	15	26

Table A.1. Continued.

Zone	Site	02/02/06	04/04/06	06/07/06	07/19/06	08/22/06
Near Shore	LM/A-S			2	2	2
	LM/A-B	X	X	X	X	X
	LM/B-S					
	LM/B-B					2
	LM/C-S	2			2	
	LM/C-B					
	LM/CD-S					2
	LM/CD-B	1				
	LM/D-S					
	LM/D-B					2
	LM/E-S	2				2
	LM/E-B	1			2	
	CASE	1				2
	CLKS-S		1		1	2
	CLKS-B	X	X	X	X	2
Powderhorn Lake	PL/A-S	1			2	2
	PL/A-B				2	
	PL/B-S				2	2
	PL/B-B				1	
	PL/C-S				2	2
	PL/C-B			2	3	1
	PL/D-S					
	PL/D-B		X		2	
	PL/E-S		X	2		1
	PL/E-B	X	X	X		X
Bay	SO/01-S					
	SO/01-B					
	SO/02-S	1				
	SO/02-B					
	SO/03-S					
	SO/03-B	2				
	SO/04-S	1				2
	SO/04-B				1	
	SO/05-S	1				2
	SO/05-B					
	SO/06-S					1
	SO/06-B					
Sites + for *V. vulnificus*		10	1	3	12	16

Table A.1. Continued.

Zone	Site	09/20/06	10/18/06
Near Shore	LM/A-S		
	LM/A-B	X	X
	LM/B-S		2
	LM/B-B		
	LM/C-S		2
	LM/C-B		2
	LM/CD-S		2
	LM/CD-B		1
	LM/D-S		
	LM/D-B		1
	LM/E-S	2	
	LM/E-B	1	1
	CASE		1
	CLKS-S		1
	CLKS-B	X	X
Powderhorn Lake	PL/A-S		
	PL/A-B		2
	PL/B-S		2
	PL/B-B		2
	PL/C-S		
	PL/C-B		2
	PL/D-S		
	PL/D-B		
	PL/E-S		
	PL/E-B	X	
Bay	SO/01-S	1	2
	SO/01-B	2	
	SO/02-S	2	
	SO/02-B		2
	SO/03-S	2	
	SO/03-B		
	SO/04-S		2
	SO/04-B		1
	SO/05-S		2
	SO/05-B		
	SO/06-S		1
	SO/06-B		
Sites + for *V. vulnificus*		6	19

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